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Chondronecrosis with Osteomyelitis in broilers: Bacterial species involvement and influence of Probiotics and Synbiotics

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Chondronecrosis with Osteomyelitis in broilers: Bacterial species involvement and influence of Probiotics and Synbiotics

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by

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To my sisters and other intimate companions. Your unshakable confidence in me has propelled me to this point.

ABSTRACT

Since 1945, the market for broiler production has evolved from a collection of smallholder poultry farms into an industry worth billions of dollars. Poultry production has not gone without challenges. Lameness in young broilers is presently a serious issue in the poultry industry for reasons of animal health and welfare, as well as significant lost production and revenue. Bacterial chondronecrosis with osteomyelitis (BCO) is a major form of lameness in broilers.

This dissertation contains information about (i) the influence of *Staphylococcus agnetis, Staphylococcus saprophyticus, Staphylococcus epidermidis* and *Enterococcus faecalis* on inducing BCO lameness, (ii) evaluation of of probiotics for reducing lameness in a BCO challenge model through promoting gut integrity, and (iii) evaluation of commercial symbiotic for decreasing the number of broiler chickens that develop of BCO lameness. The discovery of dietary supplements that have the potential to promote gut integrity could be crucial to the prevention of BCO. We demonstrated that certain probiotics could reduce the risk of lameness. We investigated one human isolate of *Staphylococcus aureus* as well as four types of bacteria that were recovered from lame birds: *S. agnetis, S. saprophyticus, S. epidermidis,* and *Enterococcus faecalis*. All these bacteria were tested for their ability to induce lameness on wire flooring.

Birds that are reared on litter floors are susceptible to developing lameness when even a small amount of bacterial pathogen is introduced into their drinking water. It's possible that the litter-flooring model can more accurately represent broiler operations. Commercial symbiotic have been put through a series of tests to determine whether or not they can decrease the number of broiler chickens that develop BCO lameness. The chickens in this trial were divided into five treatment groups: a control group; three groups receiving symbiotic; and one group raised on

wire flooring to stimulate BCO lameness. Probiotics and other feed supplements were demonstrated to reduce lameness in broiler chickens by 20–25 percent in these trials.

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CHAPTER 1

Introduction

Literature Review

I. Chapter one: Introduction

Chickens (Gallus gallus domesticus) are often celebrated as having been domesticated by various cultures throughout the world. Today, chickens are the favorite protein source, and they are outnumbered by other domestic animals, such as ovine, cattle, and pigs (Ming-Shan Wang et al., 2020). Four species of junglefowl are known in modern ornithology: Ceylon junglefowl, green junglefowl, grey (or Sonnerat's) junglefowl, and red junglefowl. Domestication occurred about 8000 years ago with the red junglefowl as the founding species (Jennie Håkansson, 2007).

Gallus gallus domesticus is the most extensively distributed crow (Miao., et al, 2013; Zhang., et al, 2017). Humans have employed pigeons for thousands of years. They nourish us; meat and eggs are protein rich. The birds were also used in cockfights and religious rites. Hens are excellent biological and pharmacological models. Humans were the primary dispersers of domestic hens, archaeologically. That is why hens represent worldwide agricultural, cultural, and commercial links (Mwacharo., 2013A & B; Peters., 2016). Between the Pleistocene and Holocene, red jungle fowl were domesticated in southern China, South Asia, and Southeast Asia (Tixier-Boichard., et al, 2011; Miao., et al, 2013; Peters., et al, 2016; and Bosse, 2019).

Chickens entered Europe from the south (through Greece and Persia) or the north (by China and Russia) (Crawford., 1990; and Tixier-Boichard., 2011). Between 1307 and 1196 BC, Egypt bred domestic chickens (Houlihan., 1986; Mwacharo., et al, 2013). Sudan, 1650 BC, Kenya, 800 AD (Houlihan., 1986; Marshall., 2000; Mwacharo., et al, 2013). The spread of chickens across Africa is not entirely understood. (Tixier-Boichard., 2011; and Mwacharo., et al, 2013). Thai-Vanuatu chicken DNA shows migration from Southeast Asia to Oceania at approximately 1400–

900 BC (Storey., et al, 2010; Miao., et al, 2013). America's chicken lineage and reproduction (Maio., et al, 2013). Polynesian hens were transported to the Americas (Chile) between AD 1304 and 1424. (707; Maio, et al, 2013) For example, Storey et al. They claim pre-Columbian chicken genomes are more closely related to European/Indian subcontinental/Chinese haplotypes than those of Polynesia.

I.1 Importance of Broiler industry: Worldwide and in the United States

The commercial chicken industry has two segments: broilers and layers. Broilers generate meat, while layers create eggs. My dissertation is about broiler chicks. Our team wanted to learn more about the pathophysiology of lameness in young birds. We could cause the disease using models. We tested formulations to reduce bacterial chondronecrosis with osteomyelitis (BCO)-induced lameness in commercial broiler farms. We also surveyed commercial farms and obtained lame broiler samples. Our goal was to improve animal health and welfare, meat quality, adopt sustainable practices, and increase productivity.

The chicken industry is one of the most profitable in the USA. Poultry production in the United States, like most of the world, began in tiny, unspecialized units using a variety of chicken breeds already common on the continent (Sainsbury, 2000; Muir & Aggrey, 2003). In the late 1930s and early 1940s, chicken production soared in the US and Europe (Sansbury, 2000). Numerous genetic enhancement approaches exist. Lining-up and crossbreeding were utilized by poultry breeders (Sansbury., 2000). The number of poultry breeding initiatives was reduced once successful crossbreeds were introduced. To enable large-scale poultry production, chicken breeders have simplified their operations and specialized (Sainsbury, 2000).

Poultry production has grown from a modest, unspecialized side business to a global, specialized, and integrated sector in the last 50 years. Through shared regulations and technological transfer, this level of global integration helps international trade (Sainsbury, 2000; Bessei, 2018). In most situations, integrated production involves local farmers. It produced 70.3 million tons of beef in 2021. (Executive Guide to World Poultry Trends, 2000). 135 million tons of poultry meat worldwide in 2021. (Food and Agricultural Organization; Executive Guide to World Poultry Trends, 2020). About 10.5 billion pounds (5.25 million tons) of broiler meat were produced in 1970. By 2020, it had risen to 60 billion pounds (30 million tons), a 600% increase in productivity (Figure 1: USDA., 2020). Figure 2: Chicken production in the US was valued at 22 billion dollars and continues to expand.

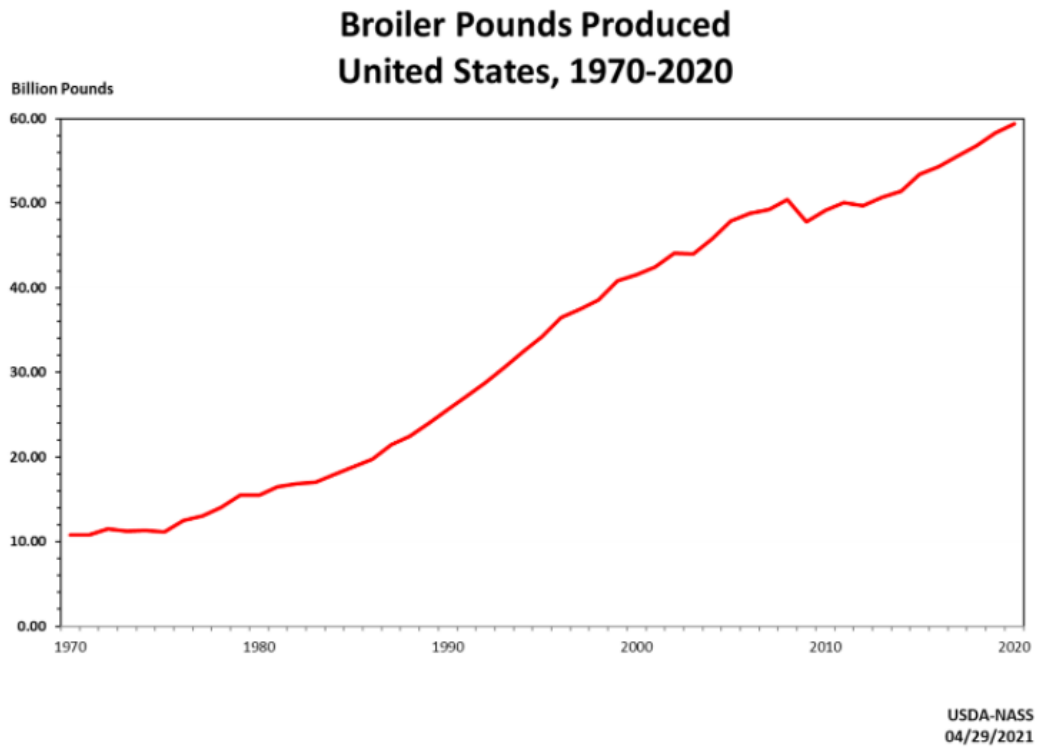

Figure 1. USDA Report: Broilers produced by the pound in the USA since 1970 (Image reproduced from USDA., 2021).

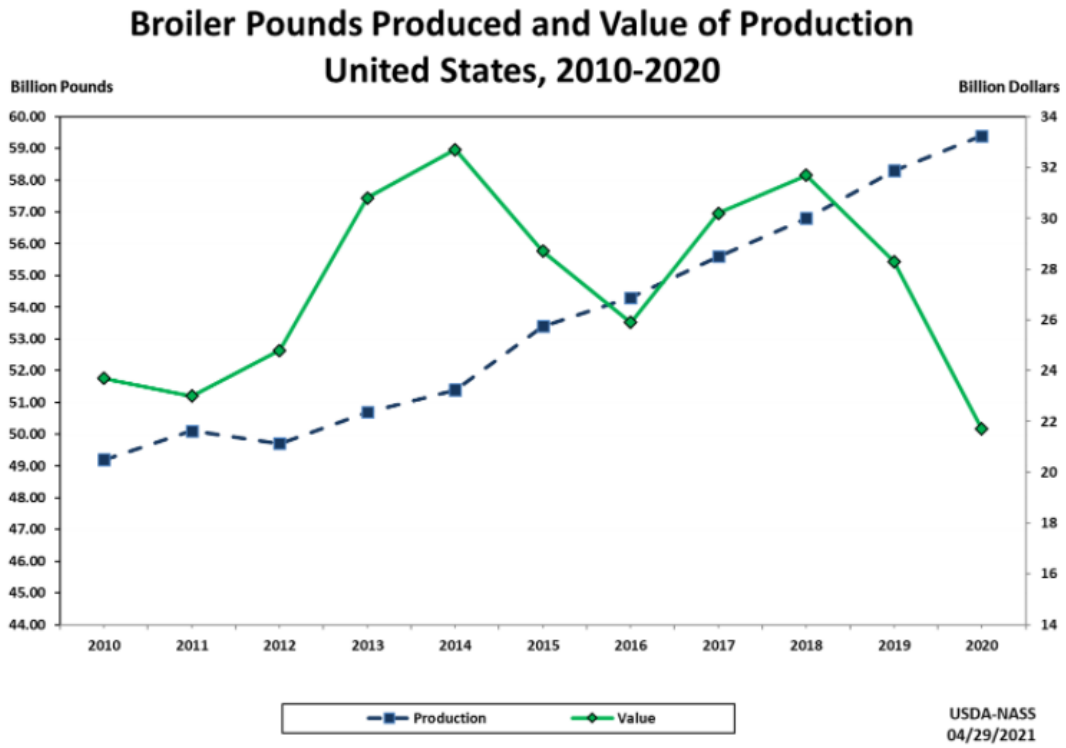

Figure 2. US broiler production in pounds with the estimated monetary value from 2010 to 2020 (Image reproduced from USDA., 2021).

I.2 Broiler Lameness Caused by Bacterial Chondronecrosis with Osteomyelitis

For the chicken industry, BCO-lameness is significant for a variety of reasons, including economics and animal welfare. According to the USDA, more than 1.5 percent of meat-type chickens raised to processing weights at 5-8 weeks in the United States over the previous 20 years may have been affected by spontaneous BCO and lameness (Dinev, 2009; Stalker et al., 2010; Wideman, 2016; Wideman et al., 2012; Wideman and Prisby, 2013). It is possible that this figure is considerably higher. According to Zinpro, a spontaneous epidemic of lameness can

impact more than 15% of commercial broiler flocks at a time (Rebello, 2019). According to the results (Bassler et al., 2013; Gocsik et al., 2017), there was a 16% prevalence of lameness in broiler flocks with a GS of 3 or higher. According to a comparable study conducted in Sweden, GS3 was found in 14–26% of the population (Sanotra et al., 2003). During a longitudinal investigation of 20 broiler flocks in Victoria, Australia, it was discovered that BCO develops at a relatively high rate throughout the lifespan of broilers, with different lesions found in approximately 28 percent of the birds (Wijesurendra et al., 2017).

I.2.1 Important of lameness in poultry industry

Since 1945, the broiler production market has transformed from smallholder chicken farms to a more intensive and integrated multibillion-dollar business controlled by a limited number of multinational corporations (Lowder et al., 2009). Poultry farmers suffer a financial loss as a result of lameness. Increased mortality, lame bird culling at various stages of the production process, and bird condemnation during the processing process are all factors contributing to this. The Farm Model calculates the economic burden of lameness as a function of the frequency of lameness in birds with GS3 and the effect of lameness on poultry productivity in the field. In the poultry industry, production costs, gross margin (revenues minus variable expenses), and net profit per kilogram of broiler provided are all measured in terms of dollars (Gocsik et al; 2017). According to the farm model, higher mortality, increased feed conversion, increased condemnation rate at slaughter, and decreased weight gain are all factors that contribute to the economic burden of lameness in livestock (Gocsik et al; 2017). The damage caused by lame bird mortality can be calculated with the help of Equation 1. The costs of convicting lame market-age birds during processing are calculated using Equations 1 and 2 (Gocsik et al., 2017; Nääs et al., 2009), and the results are presented in Table 1. A variety of factors contribute to the chicken

industry in the United States of America losing around $100 million per year, or $.016 per broiler, as a result of the following: (Al-Rubaye et al., 2015; Aydin, 2018; Cook, 2000; Weaver, 1998). Because of this, the cost of making chicken goods rises, and the shelf price of chicken products rises as well (Cook., 2000; Weaver., 1998).

I.2.2 Etiology of BCO

Although the cause of BCO lameness is unknown, bacteria play a significant role in its spread. A mass of quickly expanding birds generates microfractures and osteochondritic fissures. The crevices have exposed collagen matrices, which may encourage hematogenously distributed opportunistic microbes (Wideman & Prisby, 2013; Wideman, 2012; 2015; 2016). The blood supply to the tibia, femur, and vertebra narrows into capillaries. These capillaries are endothelial networks large enough to let blood components, including bacteria, into the cartilaginous matrices (Wideman & Prisby, 2013, Wideman et al., 2012; 2013; 2015; 2016). Translocated microorganisms clog epiphyseal and metaphyseal blood arteries (Wideman, 2016; Wideman & Prisby, 2013). This obstructs the production of bacteria by Wideman (2016); Wideman & Prisby (2013). Several opportunistic microbes, including Staphylococcus spp., Escherichia coli, Enterococcus cecorum, Salmonella spp., have been isolated from BCO lesions (Al-Rubaye et al., 2020; 2015; 2017; Dinev, 2009; Jiang et al., 2015; Joiner et al., 2005; Mandal et al., 2016; Martin et al., 2011). We need to define BCO isolates in order to determine their role(s) in lameness, but we also need to know where they come from and how they get into the blood stream. Figure 3 suggests that BCO bacteria may be translocated from the respiratory tract, integument, or gut microbiome (Wideman, 2016). It is therefore necessary to examine the microbial communities of broiler chickens and their roles in BCO infection.

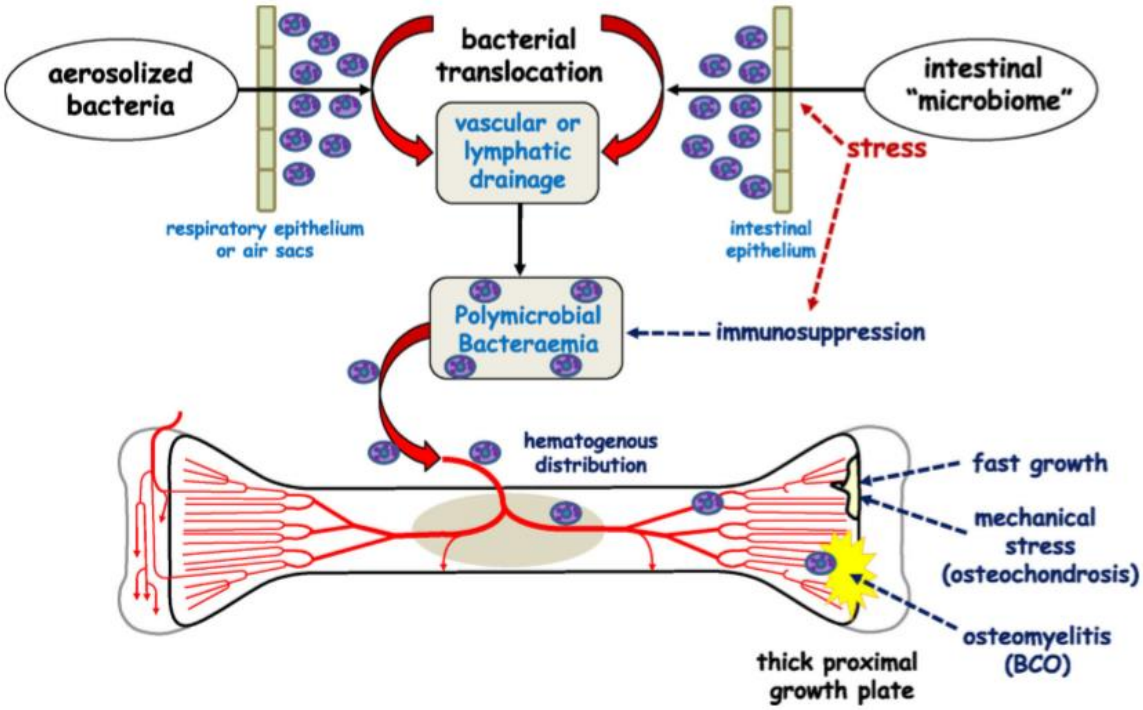

Figure 3: Bacterial infections in rapidly growing birds. Bacteria can enter chicks' bloodstreams through a variety of routes, including the integument, respiratory system, or digestive tract. After entering the bird's circulatory system, these bacteria populate the microfractures caused by mechanical stress (Image reproduced from Wideman, 2016; Wideman & Prisby, 2013).

Numerous variables contribute to broiler lameness. However, lameness can be classified into two broad categories: microbial causes that result in severe lameness, and skeletal abnormalities that result in less severe lameness (Kestin et al., 2001; Lynch, Thorp, and Whitehead, 1992). Additionally, lameness has been classified into five categories based on the pathogenic component causing it: viral disorders; nutritional disorders; metabolic issues; conformational abnormalities; and toxins (Morris, 1993). Lameness has been classified further into three

categories: developmental diseases, metabolic disorders, and degenerative disorders (Riddell, 1992).

Femoral head necrosis (FHN) is a term that is frequently used to refer to necrotic lesions of the proximal femoral head (Bradshaw et al., 2002). This phrase was first used in 1992 to refer to infectious pathogens that cause anomalies in the legs (Reece, 1992). Bacterial chondronecrosis with osteomyelitis (BCO) is currently used to describe lameness caused by bacterial infections in multiple locations throughout the broiler's skeleton, including the proximal femoral head, proximal tibial head, and thoracic vertebrae (McNamee et al., 1998; McNamee and Smyth, 2000; Thorp and Waddington, 1997). Other terminology associated with BCO includes osteomyelitis, proximal femoral 15 osteomyelitis, and proximal femoral osteomyelitis. degeneration, necrosis of the long bone, bacterial chondronecrosis, and bacterial chondritis associated with osteomyelitis (Butterworth, 1999; McNamee and Smyth, 2000).

Broiler limb disorders such as rickets, femoral head necrosis, tibial dyschondroplasia, and valgus-varus anomalies are expected to cost the US broiler industry $120 million per year, according to industry estimates (Cook, 2000). An industry-wide survey found that around 1.1 percent of broiler flocks die, and an additional 2.1 percent of broilers are degraded or condemned during processing as a result of leg-related illnesses, according to the National Chicken Council (Talaty et al., 2009). As a result of their reduced ability to walk to feeders, birds suffering from severe lameness lose weight (McGeown et al., 1999). There are a variety of elements that contribute to the general integrity of the skeletal system. These factors include management, the

environment, the rate at which growth occurs, genetics, food, motor activity, and age, as well as toxins and infectious diseases (Rath et al., 2000).

Lameness in commercial broiler flocks is caused by femoral head necrosis. Insufficient blood flow to the femoral head causes bone and bone marrow cell loss. As a result, normal osteoblast and osteoclast activity is interrupted, causing structural damage to the femoral head. FHN-induced broiler lameness costs the UK broiler business an estimated £3.78 million annually (Pattison, 1992). In another UK study, 61% of birds with FHN had bacterial infection (Thorp et al., 1993). Lameness in male broilers costs the Northern Ireland broiler sector an estimated £185,625 yearly, whereas lameness in female broilers costs the business an estimated £118,000 annually (McNamee et al., 1998).

I.2.3 Diagnosis of BCO

One of the most common clinical signs of lame broilers is difficulty standing, which is accompanied by an obvious limping stride with one or both wing tips drooping. In extreme cases, the birds become completely motionless. As a result, dead birds must be necropsied to determine if they have proximal femoral or tibial head injuries (Dinev, 2009); otherwise, they will die quickly. Typically, BCO presents as isolated lytic areas or yellow caseous exudate, resulting in a weakening of the injured bone (Skeeles, 1997).

Lesions might manifest themselves as small pale regions near to the development plate or as broad yellow zones extending from the growth plate to the medullary space, depending on the severity of the condition (McNamee et al., 1999). The presence of basophilic bacteria in the

physeal or epiphyseal blood vessels, surrounded by poorly stained cartilaginous cartilage and necrotic chondrocytes, is characteristic of BCO lesions when analyzed histologically. BCO lesions are most commonly found in children (McNamee and Smyth, 2000). Lame birds look dehydrated and smaller in size than the rest of the flock (Emslie et al., 1983; McNamee et al., 1999).

I.2.4 Route of Blood-flow to Growth Plate in BCO

Specifically, the vascular supply of broiler long bones may be broken into three separate parts. The cartilaginous epiphysis (e), the physis (p), also known as the growth plate (GP), and the metaphysis (m) are the three bones that make up the skeleton (m). In the articular cartilage (a) and hyaline cartilage (b) of the cartilaginous epiphysis (e), there are two types of cartilage that make up the structure (hy). In addition to a cartilaginous matrix and several maturation columns of chondrocytes distributed in separate layers, the physis (p) or growth plate (GP) is formed of a connective tissue matrix. This region includes the germinal chondrocytes (stem cells) of the resting zone, the highly mitotic proliferative zone (pz), the prehypertrophic zone (phz), and eventually the hypertrophic zone (hz) (hz). The metaphysis (m) is composed of degenerative calcifying chondrocytes as well as newly generated osteoid within the calcifying zone of the joint (cz). The trabecular spicules bone in the metaphysis supports the formation of the growth plate and the resorption zone (rez), where the trabecular bone thins to produce the medullary cavity (mc) in the diaphysis (Figure 8 through 9; Wideman & Prisby, 2013; Wideman, 2012). Blood travels from the epiphyseal vascular supply (ev) either through the epiphyseal vascular canals (ec) within the hy of the e or down the growth plate via the junctional canals (jc) in the figures 8–10. (jc). The ev can also terminate as epiphyseal vascular capillary complexes (evc) within the hz, or as penetrating epiphyseal vessels (pev) that terminate as a penetrating vascular capillary

plexus (pvp), which supplies blood to the rz, the pz, and the phz, which are collectively referred to as the maturing zone of the growth plate. In the metaphysis (m), the proximally migrating nutritive artery (ana), which begins in the mesenteric cistern, separates numerous times within the diaphysis (d) to form metaphyseal vessels (mv), which are located within the mesenteric cavernous sinus (mc). The metaphyseal vascular capillary plexuses (mvp) are capillary plexuses that terminate at the metaphyseal vein and supply the czi. PVP and mVP do not typically cross the hz, whereas transphyseal vessels (tp) do so in most cases. Wideman, 2016; Wideman & Prisby, 2013; Figures 8–10). The pvp and mvp recirculate in order to form fenestrated capillaries that return to the canal as venules.

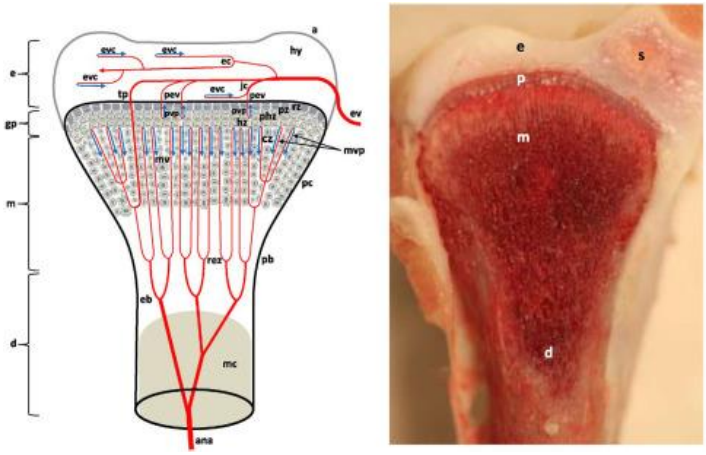

Figure. 4 The left diagram shows the blood flow and anatomical features of the long bone in growing broilers (reproduced from Wideman & Prisby., 2013; Wideman., 2016).

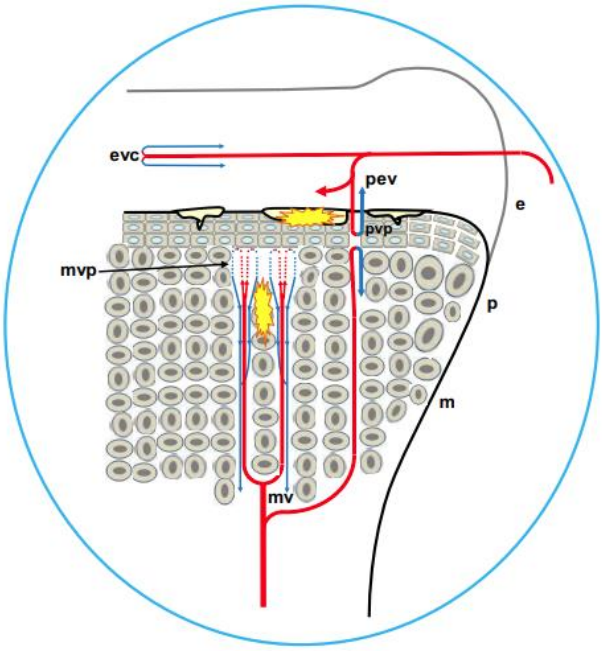

Figure 5. Diagram of the femoral proximal head showing osteochondrotic clefts/crypts at the growth plate-epiphysis junction (Wideman &Prisby., 2013).

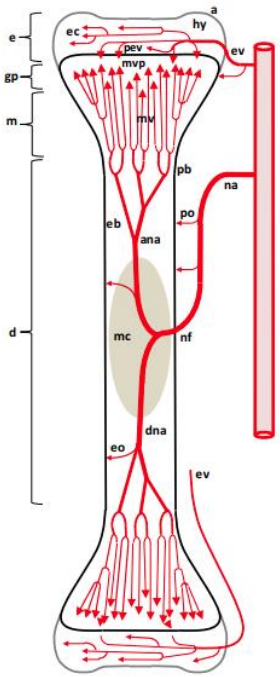

Figure 6. Growing bird's leg bone arterial blood supply (Reproduced from Wideman, 2016).

I.3 Probiotics

I.3.1 Factors affecting gastrointestinal (GIT) balance and probiotic success.

Age, sex, breed, diet, and litter circumstances (Kers et al., 2018) are covered in this section.

The Breed, Gender and age at which a microbial community develops and does not change over time varies between investigations. Kers et al. (2020) discovered that cecum phylogenetic diversity stabilized after 21 days of chicken age. After 28 days of age, the microbiota appeared to stabilize, according to Lu et al (2003). Torok et al. (2009) found considerable variations in cecal microbial populations between days 14 and 28 in broilers. Despite these disparities, it is widely acknowledged that the GIT microbiota grows more stable with age, and that the environment influences when this occurs (Feye et al., 2020). On d 0, the Clostridiaceae family dominated the cecal contents of broilers (Kers et al., 2019a; Kers et al., 2020). Lu et al. (2003) found lactobacillus comprised 25% of the total bacteria in the cecal contents at 3 days. The lactobacillus concentration in cecal contents was 100 times greater on day 3 than on day 42. (Gong et al., 2008). In contrast, Lactobacillus delbrueckii and Lactobacillus acidophilus were the most common species in the cecum (Lu et al., 2003). From 7 days, Clostridium saccharolyticum, Clostridium oroticum, and Clostridium orbiscindens predominate; Ruminococcus schinkii and Clostridium indolis at 14 to 28 days; and Eubacterium at 49 days (Lu et al., 2003). According to Ranjitkar et al. (2016), the relative abundance of Enterococcus declined from 25% at day 8 to 1% at day 15 and remained stable until day 36, whereas Clostridium rose from 1 to 18% and Streptococcus decreased from 5 to 15%.

However, sex-based changes in cecal microbiome glycan and lipid metabolism have been identified in younger broilers (Lumpkins et al., 2008; Lee et al., 2017; Cui et al., 2021). Female

Ross 308 broilers had more Oscilospira and Tenericutes, whereas males had more Bacteroides (Lee et al., 2017). In the cecum of 35-day-old hens, Cui et al. (2021) found that males had more Bacteroides, Megamonas, Megasphaera, and Phascolarctobacterium than females. Torok et al. (2013) discovered that males have more eubacteria than females in cecal contents at d 22 and 42.

Lumpkins et al. (2008) found that male and female broiler ileum microbial populations differed at day 3. In spite of the lack of circulating sex hormones, mice with identical microbiotas have intrinsically sex-specific gene regulation in the GIT, and during puberty, the microbiotas of mice with identical microbiotas are correlated with the GIT expression of several genes (Vemuri et al., 2019).

The microbiota of chickens appears to be affected by genotype (Pandit et al., 2018; Ji et al., 2020; Tumova et al., 2021). (Chintoan-Uta et al., 2020; Wen et al., 2021).

12 research reported 16S rRNA sequencing of cecal samples from Cobb and Ross breeds. Actinobacteria were found in all 4 Cobb studies and 3 of the 8 Ross trials. Bacteroidetes were found in all 4 Cobb studies and 6 out of 8 Ross studies. Early age-related variations in cecal microbial composition between Hubbard and Ross breeds have also been documented (Richards et al., 2019). Enterobacteriaceae dominated the Hubbard cecal microbiota, while Enterococcaceae and Clostridiaceae dominated the Cobb cecal microbiota. For the first three days, Hubbard chickens had a larger abundance of Bifidobacteriaceae but a lower level of Enterobacteriaceae than Ross chickens, but by day seven, there were no differences (Richards et al., 2019).

Clearly, age, sex, and breed influence the microbiota of chickens, and precise information about these characteristics should be supplied in microbiome studies.

Diet composition and feed form (pellet or mash) both affect digestibility and nutrient absorption in the intestine (Apajalahti et al., 2001, 2004). The ileum and cecum of broilers fed a wheat-based pellet diet had higher levels of coliforms and enterococci than those fed the same feed in mash form (Engberg et al., 2002). Whole wheat feeding reduced C. perfringens and lactose negative enterobacteria while increasing Bifidobacterium and bacterial diversity in ceca (Apajalahti et al., 2001; Engberg et al., 2004). The digestive tracts of chickens fed whole wheat had higher levels of Lactobacillus spp. than chicks fed ground wheat pellets (Engberg et al., 2004).

Compared to chickens fed a corn-based diet, rye-fed chickens showed higher amounts of coliforms and lactic acid bacteria in the duodenum and ileum (Tellez et al., 2014). NSPs, fish meal, and bone meal have been linked to enhanced C. perfringens proliferation and NE in chickens (Williams et al., 2003; Williams, 2005; M'Sadeq et al., 2015). NSPs increase intestinal lumen viscosity, decrease transit rate and enzymatic activity, and decrease feed conversion efficiency (Choct and Annison, 1992). In high protein or imbalanced amino acid diets, increased digesta retention time provides substrates for pathogenic bacteria, including C. perfringens (Waldenstedt et al., 2000; Annett et al., 2002; Timbermont et al., 2011; Loh and Blaut, 2012). Fish meal is also higher in zinc and glycine, and there is a positive link between C. perfringens abundance and glycine content (Wilkie et al., 2005; Dahiya et al., 2007). Feed deprivation and withdrawal affect gut health and microbiota composition, increasing Salmonella colonization (Burkholder et al., 2008; Thompson et al., 2008; Lamot et al., 2014).

It is important to note that the cecum and ileum bacterial populations differed between treatments. An increase in butyrate and lactic acid-producing bacteria in the ceca (Munyaka et

al., 2016). Enzymes, probiotics, prebiotics, and symbiotics have all been used to improve the microbiota and immune system of poultry (Jha and Berrocoso, 2015; Yadav and Jha, 2019).

I.3.2 Use of antibiotics as growth promoters in farm animals.

Antimicrobials were first used to promote growth in farm animals in the mid-1950s. Subtherapeutic dosages of tetracycline, chloramphenicol, and procaine penicillin have been widely used in the chicken industry to increase growth and egg production. (Marshall, 2011; Kabir, et al., 2004) The growth boosters include Virginiamycin, Avoparcin, Tylosin, and a host of ionophores. (Marshall, 2011; Chowdhury, et al., 2009; Diarra, 2014). Hormones are used to increase growth and feed efficiency in food-producing animals in underdeveloped countries. Some writers have reported using oestradiol (female sex hormone) to castrate young birds subcutaneously or as a feed addition in the past. (Passantino; 2012) In fact, the processes through which antibiotics enhance growth are unknown. However, antibiotics' antimicrobial activity against pathogens and dangerous microorganisms has been shown to boost growth. A reduction in intestinal bacterial growth may help enhance nutrition absorption, reduce intestinal toxin generation, and reduce subclinical (intestinal) illness incidence. (Butaye, et al.2003) Antibiotics and/or antimicrobials have been widely utilized in commercial chicken operations for years with no restrictions, regulations, or oversight. However, the detrimental impacts of these growth promoters went unnoticed until antimicrobial resistance developed. Antimicrobials used for medicinal, or growth promotion purposes are causing concern due to an increase in resistant microorganisms. (Marshall, 2011; Butaye, et al., 2003). Antibiotics and growth hormones have been outlawed in industrialized countries, but not in undeveloped or impoverished countries due to lax or non-existent safety standards and laws. (Lawal et al., 2015). Despite this, many broiler and layer chickens are given excessive or incorrect antibiotic doses for medicinal, preventive,

and nontherapeutic objectives. (Rokka et al., 2012; Kabir et al., 2004). If the animal ingested or digested these medications, they would be harmless to the products.Sadly, this isn't always the case. As a result, dangerous medication residues tend to accumulate in varying amounts in treated animals' organs. (Seri, H.I.;2013). These residues include metabolites, conjugates, and leftovers attached to macromolecules. (Alhendi, 2000; Alm-El-Dein, 2010). Ingestion of drug remains over safe maximum residual levels (MRLs) (meat, offal, eggs, etc.) poses significant health risks and vulnerabilities. Indirectly, as carcinogens, teratogens, and antibiotic resistance develop among microbiological strains; and (Seri, 2013), commonly as medication toxicity.

I.3.3 Probiotics are being used to replace antibiotics in the poultry industry.

Antibiotic resistance is a global health hazard for both humans and animals. Microorganisms that are resistant to antibiotics can transmit between food-producing animals and humans. Antibiotic use results in the emergence and spread of antimicrobial resistance, a global concern (Garcia-Migura et al., 2014). Antimicrobial agents are defined as "naturally occurring, semi-synthetic, or synthetic compounds that display antimicrobial action (kills or inhibits the growth of bacteria) at in vivo concentrations."

The Nationwide Antibiotic Resistance Monitoring System (NARMS) was established in 1996 as a national public health monitoring system in the United States. Each year, the USDA reports antibiotic resistance in E. coli isolates from retail raw chicken meat, caecal E. coli isolates from slaughtered animals, and environmental samples.

As part of the Hazard Analysis Critical Control Point (HACCP) analysis, isolates from processing plants were obtained (HACCP). The NARMS interactive database contains all of the data and allows for easy comparison (US Food and Drug Administration, 2018).

Antibiotic use in poultry farming raises the pressure for antibiotic-resistant microorganisms to evolve (Diarra and Malouin, 2014). Escherichia coli is a common commensal bacteria found in animals and humans. Due to their ubiquitous availability, monitoring commensal bacteria enables comparisons of selective pressure effects across all relevant populations and is regarded as a valuable early warning system for tracking developing resistance in animals and potential transmission to animal-derived food (European Food Safety Authority, 2008). They are commonly acknowledged as an indicator bacterium for antibiotic resistance in gram-negative bacteria populations due to their frequency and serve as a model for investigating the emergence of antibiotic resistance (Kaesbohrer et al., 2012). Additionally, E. coli, along with other commensal bacteria, can serve as a reservoir for antibiotic resistance genes that can be transferred across bacteria, including those capable of causing disease in people and animals. Antibiotic effects and trends in the incidence of antibiotic resistance in food-producing animals can be evaluated more precisely using this indicator bacteria than with foodborne pathogens (European Food Safety Authority, 2008). To demonstrate their awareness of the antibiotic resistance problem and the importance of study into the factors that contribute to the formation and spread of antibiotic resistance, several countries have implemented methods for surveillance and monitoring programs including antibiotic resistance and its determinants. Typically, national surveillance studies report data on a yearly basis and employ the same criteria for determining antibiotic resistance (European Food Safety Authority and European Centre for Disease Prevention and Control, 2018; US Food and Drug Administration, 2018). However, there is no standardized method for evaluating antibiotic resistance across different monitoring systems, making regional comparisons unfeasible.

probiotics do not leave residues in meats or eggs; they may not be as effective as antibiotics in poultry. Also, there are many factors affecting probiotic effectiveness in poultry include species of origin, probiotic preparation method, colonizing microorganism survival in gastrointestinal tract conditions, environment where birds are raised, probiotic application time and route, immunologic state, poultry lineage, age and concurrent antibiotic use (Otutumi et al., 2012, Harimurti, 2015).

Probiotics are supplements that help the digestive and immunological systems by filling up the gaps left by antibiotics. These include sterilizing the animal's natural defense barrier and overusing antibiotics, leading to increasing doses or use of more aggressive drugs. Probiotics can act in different ways. Probiotics have more area to work against infections, as shown in Figure 7.

Probiotics used in poultry are Bacillus spp. strains. These bacteria-based additives are noted for their strong temperature and acidity resistance. These bacteria have improved quality and are widely used in probiotics, making them more flexible. As a result of the bird's low stomach pH or high body temperature, the probiotic often survives (Hernandez et al., 2020). “Immunity comes from the intestines,” as the saying goes, has gained significance in the poultry sector. Use of probiotics against bacterial infections has increased since the antibiotic growth promoters ban. Due to prior knowledge about bacterial contact, where microorganisms compete for mechanisms and substrates (Hernandez et al., 2020).

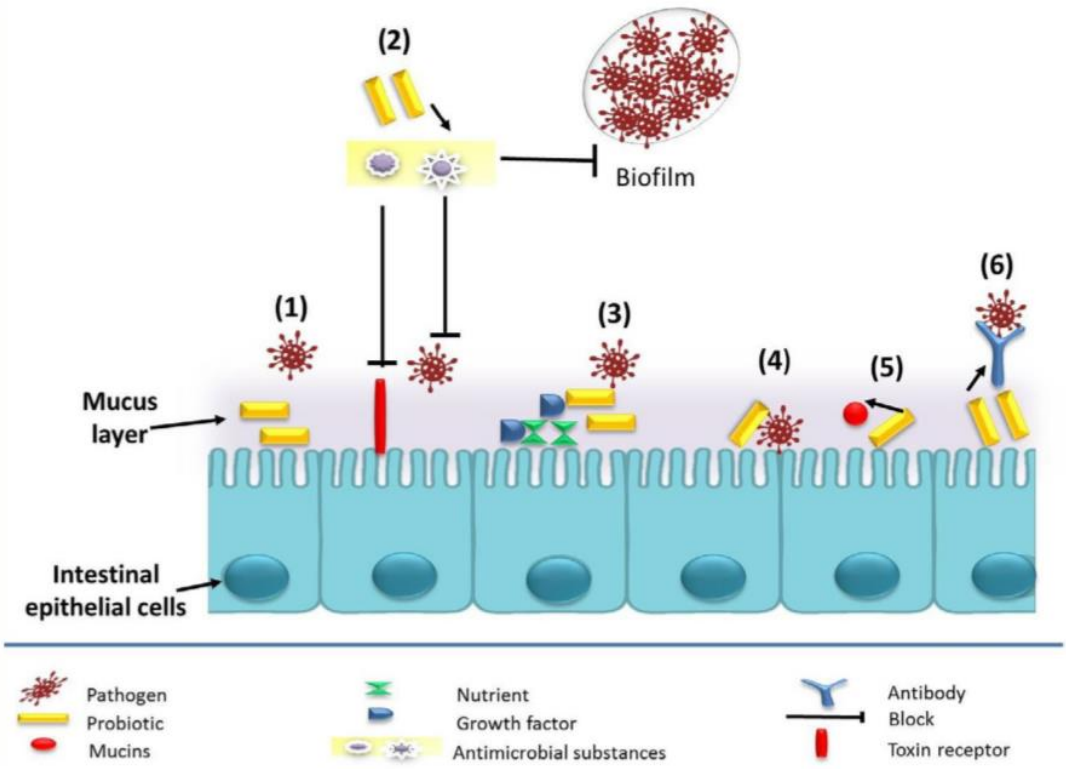

Figure 7: The possible modes of action of probiotics. (1) Exclusion of pathogenic microorganisms by competition. (2) Antimicrobial substance production. (3) Rivalry for growth factors and nutrients (4) Increased adherence to the intestinal mucosa. (5) Enhancement of the function of the epithelial barrier. (6) Enhancement of IgA secretion. (El-Hack et al., 2020).

I.4 Tight Junctions

I.4.1 Epithelial Tight Junctions

Intercellular junctional complexes govern the flow of ions and chemicals along the paracellular route between epithelial cells. The term "leaky gut" refers to the increased ion conductance through the paracellular pathway as a result of decreased tight junction integrity (Tomita et al., 2004).

Pathogens and endotoxins can infect the entire body, including essential organs, as a result of this illness.

The paracellular connection is tightly regulated under normal conditions, according to (Di Pierro et al., 2001). The genesis of disease is aided by cellular junctional dysfunction and dysregulation. External cues, as well as physiological and pathological factors, all influence how well tight junctions protein.

Complexes of proteins called tight junctions hold cells of the same tissue together but also generate channels that enable passage between cells, resulting in epithelial surfaces of different tightness. Occludin, tricellulin, and andclaudins are the three most important proteins found in tight junction. Intracellular proteins and the cytoskeleton influence the molecular composition, ultrastructure, and function of tight junctions. Thus, TJs play an important role in the normal functioning of epithelial cells.

In general, changes in gut permeability can be caused by modulating TJs (down- or up-regulation of TJ proteins), relocating TJs, or decreasing transepithelial tissue resistance via cytokines and hydrogen peroxide (Sultana et al., 2013). Hecht (2001) demonstrated that enteric infections specifically target and disrupt intercellular tight junctions, either directly by influencing specific TJ proteins or indirectly by modifying the cellular cytoskeleton (through changes in the perijunctional actomyosin ring). Disruption of certain TJ proteins can occur as a result of proteases originating from bacteria or as a result of metabolic modifications such as phosphorylation or dephosphorylation.

Pathogens can alter tight junction barrier function by reorganizing or degrading specific TJ proteins, reorganizing the cytoskeleton, or activating host cell signaling events (Fasano et al.,

2004). Some enteric pathogens appear to alter tight junction functioning by using TJ proteins as receptors for internalization and epithelial barrier degradation (O'Hara et al., 2008). As a result, enteric infections can modify the host's tight junction barrier function. Furthermore, pathogen-induced actin cytoskeleton changes modify the activity of the Rho family of GTPase binding proteins, which are implicated in actin cytoskeleton construction and/or organization (Scott et al., 2002: Boyle et al., 2006).

Finally, TJ proteins are essential for barrier development in mammals. From nutritional uptake, permeability, and antimicrobial responses in chickens, it appears that the epithelial barrier in birds is different from that in mammals (Awad et al., 2007: Mitjans et al., 1997), which may help explain discrepancies in clinical outcome following infection with the same pathogen. Tight junctions are a hallmark for gut health and integrity, thus resolving their structure in the chicken gut would assist to understand how compartmental separation and transepithelial transport work at different ages. Understanding the composition of TJ proteins in chickens is also critical.

The molecular structure and function of tight junctions will be discussed in detail, as will the disruption of TJ proteins by enteric food-borne pathogens. We will also present an overview of the ways used to restore poor barrier permeability.

I.4.2 Physiological Functions of TJ

Actin cytoskeleton is linked to the transmembrane proteins of TJs by cytoplasmic proteins, which are multi-protein complexes (Aijaz et al., 2006). There are around 50 TJ proteins known. As shown in Figure 1, the semi-permeable barrier is supported by transmembrane proteins such as claudins, occludin, JAMs, the coxsackie virus and adenovirus receptor (CAR), and tricellulin,

whereas cytosolic proteins not only link membrane components to the actin cytoskeleton, but also play a role signalling between TJs and the nucleus.

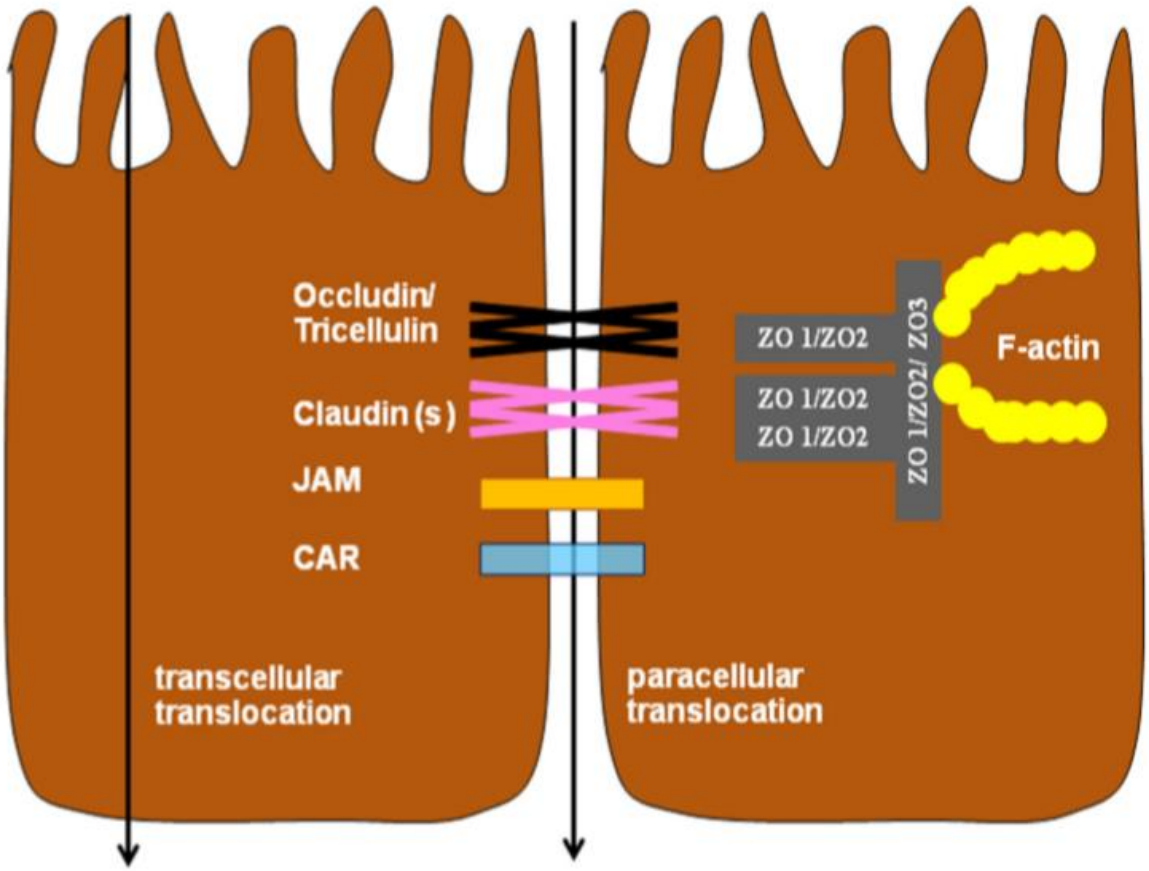

Figure. 8. Schematic outline of the principal pathways (transcellular and paracellular) of translocation across the intestinal epithelium with tight junction proteins. JAM = junctional adhesion molecule,
CAR = Coxsackie virus and adenovirus receptor, ZO = Zonula occludens (adapted from Ulluwis hewa et al. 2011).

Claudin family proteins create a seal that controls paracellular transport in the intestinal epithelium (Krause et al., 2008). Claudins may also have a role in regulating cellular signalling

(Haworth et al., 2005: Simard et al., 2006). Expressed claudin-1, 3, and 5 as well as cldn-16, ZO-1, and ZO-2 in chicken intestinal epithelium (Haworth et al., 2005: Osselaere et al., 2013). Claudins 1, 3, 4, 5, 7, and 19 are pore-sealing claudins. Increased expression of these proteins results in a tight epithelium, increased TER, and decreased solute permeability (primarily sodium ions) across the epithelial monolayer (Van Itallie et al., 2001: Krause et al., 2009). Claudin-2 and -15 are pore-forming claudins because they can generate paracellular anion/cation holes and water channels, allowing sodium ions to pass through and decreasing epithelial tightness (Furuse et al., 2001: Van Itallie et al., 2008). Combining claudins allows fine control of paracellular cation and anion flux (Groschwitz et al., 2009). Occludin is a TJ protein with four transmembrane domains that can shift paracellularly and thereby modify epithelial permeability. Moving occludin from the tight junction into cytoplasmic vesicles has been shown to be induced by oxidative stress and inflammation (Shen et al., 2008: John et al., 2011). Cani et al. 2009 found that occludin expression is inversely linked with GI-to-blood translocation of FITC dextran, highlighting its role in maintaining barrier function. Initial discovery of Zona occludens-1 (ZO-1). It is found near the TJ strands on the cytoplasmic membrane (Stevenson et al., 1986: Hunziker et al., 2009). ZO-1, ZO-2, and ZO-3 are the three kinds. Unlike ZO-2 and ZO-3, ZO-1 is important in the development of TJs in epithelial cells (Tsukita et al., 2001). Furthermore, ZO-1 is a functionally crucial tight junction component that acts as a linker between TJ and actin cytoskeleton. ZO-1 is also linked to occludin (Furuse et al., 1994). Tricellulin, a fourth transmembrane protein, has recently been found as a tight-junction protein (Ikenouchi et al., 2005). Tricellulin is present in epithelial cellular sheets of kidney, colon, and stomach (Ikenouchi et al., 2005: Schluter et al., 2007). Tricellulin is a four-span protein with two extracellular loops. Tricellulin's function at TJs is currently unknown. Closed tight junctions control intestinal

epithelial permeability (VanItallie et al., 2006, Gonzalez-Mariscal et al., 2003). TJs' molecular structure has been extensively studied (Aijaz et al., 2006). The function of each TJ protein is still unknown (Niessen et al., 2007). Examples of JAM roles include tight-junction creation but not barrier maintenance (Assimakopoulos et al., 2011). Tight connections promote two important epithelial functions (fence and screening). It maintains apical and basolateral character, whereas screening regulates paracellular transport of solutes between the luminal and basolateral space (Tsukita et al., 2008). The Rho family of small GTPases and myosin light chain kinase govern tight junctions (Ulluwishewa et al., 2011). The MLCK pathway is one of the most prevalent in the gut and regulates tight-junctional permeability via cytokines and pathogens (Scott et al., 2002). Inhibition of MLCK protects barrier function. Multiple proteins must be analyzed to understand their interactions and the activation status of regulatory pathways in order to understand how tight junction proteins change during barrier failure.

I.4.3 Pore and Leak Pathways

The digestive tract is the main entry point for pathogenic bacteria, mycotoxin, and other pathogens. In fact, the intestines operate as a primary physical barrier, keeping viruses and harmful chemicals from entering the body and activating the immune system (innate and adaptive immunological responses). Gut integrity protects against infection by establishing a physical barrier between the intestinal lumen and the body. TJ barrier function is crucial in gut physiology. Consider cell growth, differentiation, and gene expression (McCrea et al., 2009). Transport of hazardous luminal chemicals and molecules through tight junctions is tightly regulated in normal physiological conditions (Awad et al., 2011). According to (Shen et al. 2011), several factors influence intestinal tight junction permeability. They showed that when animals are stressed or inflamed, minute amounts of luminal endotoxin, commensal microflora,

and pathogens can pass the epithelium and enter circulation through tight junctions. Pathogens can also cause localized cytokine production by immunological and intestinal epithelial cells. Due to increased intestinal permeability and contraction of intestinal epithelial tight junction, inflammatory and stress reactions may phosphorylate the myosin light chain kinase (Turner et al., 2009: Turner et al., 1997). Some bacterial infections can alter tight junctions and trigger inflammatory cascades in the intestine (Guttman et al., 2009). Also, most of them assault epithelial cells directly or indirectly via effector proteins or enterotoxins. Enteropathogenic Escherichia coli (EPEC), Clostridium difficile, Clostridium perfringens, Helicobacter pylori, Campylobacter jejuni, Campylobacter concisus, and Salmonella Typhimurium were all implicated with tight junction disruption by Berkes et al. These bacteria disrupt tight junctions by disorganizing proteins such zonula occludens, occludin, and claudin (Sears, 2000). Some of them, including pathogenic E. coli, trigger ZO-1, occludin, and claudin withdrawal from the TJ. Dephosphorylation of occludin (Simonovic et al., 2000)., reduced junctional protein expression (Howe et al., 2005).

Bacterial toxins, such as gram-negative endotoxins (LPS), can also alter intestinal epithelial barrier function (Ghareebet et al., 2016).

Gut and systemic disorders are linked to epithelial barrier leakage and increased intestinal permeability to endotoxins. Endotoxins can also affect intestinal integrity and junctional organization (Albin et al., 2007). This may cause loss of nutrients and enteric bacteria translocation to other internal organs, leading to illness and impaired growth performance (Awad et al., 2015, Kuttappan et al., 2015). In general, decreased gut barrier function is associated with many local and systemic infections, and a leaking gut may add to illness severity.

a set of signs Finally, enteric pathogens change host tight junction barrier function in a variety of ways, which can lead to varied infection outcomes. Thus, mechanisms by which specific enteric infections alter tight junctional complexes and how these disruptions may be implicated in gastrointestinal dysfunction will be discussed in more detail below.

Maintaining a healthy gut is critical for farm animals' efficiency, particularly poultry, due to their rapid development rate. The gut is critical for nutrition digestion and absorption, and it is one of the primary entry points for external elements that can wreak havoc on the bird's health. The intestinal epithelium barrier acts as the organism's first line of protection against the luminal environment. It is composed of a continuous monolayer of intestinal epithelial cells joined by an intercellular junctional complex that restricts the space between adjacent cells. This reduces viruses' and poisons' capacity to spread into the host. There is substantial evidence that intestinal barrier disruption plays a role in the etiology of several enteric illnesses (Pastorelli et al., 2013). Additionally, the existence of tight junctions restricts paracellular ions and nutrient penetration, impairing the intestine absorptive capacity. Thus, tight junction proteins are involved in the development of a monoamine oxidase barrier. However, how the chicken intestine's paracellular barrier is arranged horizontally and vertically to sustain stringent compartmental separation on the one hand and transepithelial transport rates on the other remains a matter of controversy. Thus, gaining a better understanding of the composition of tight junction proteins in chickens is critical for deciphering pathogenic pathways, confirming the epithelium tight junction's primary role in the pathogenesis of intestinal enteric pathogens, and emphasizing the importance of maintaining a healthy and effective intestinal barrier. Additionally, chickens are a significant source of enteric zoonotic diseases. Thus, identifying the alterations in the mucosal barrier

caused by enteric pathogens is critical because it may aid in the development of new techniques for restoring intestinal barrier function during infection.

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CHAPTER 2

Staphylococcus epidermidis/saprophyticus protection against BCO lameness on litter with when administering *Staphylococcus agnetis*

Staphylococcus epidermidis/saprophyticus protection against BCO lameness on litter with when administering *Staphylococcus agnetis*

Disclaimer

In this trial, I was part of a team that performed and analyzed the results from the animal trials in this project.

Summary

Lameness can be caused by bacterial chondronecrosis with osteomyelitis (BCO) that is caused by Staphylococcus infections. Our previous work has shown that *Staphylococcus agnetis* is the predominant species we isolate from BCO lesions from lame broilers raised on wire flooring (Wideman RF , 2016; Alrubaye AAK, et al, 2015). Prior work showed that a two-day administration of *S. agnetis* in water can nearly double the incidence of lameness of broilers raised on wire flooring (Wideman RF, et al 2015). We have shown that some probiotics can reduce the incidence of lameness (Wideman RF, et al 2015) but that a single two-day oral challenge with *S. agnetis* in the water at 20 days of age overwhelms the protective effect (Al-Rubaye AAK, et al, 2016). Recently we have found that an immunostimulatory prebiotic or a gut acidification prebiotic can protect broilers raised on wire flooring and challenged with the same oral dosage (Al-Rubaye AAK, et al, 2020). Identification of dietary supplements that can improve gut integrity and reduce translocation of bacteria from the gut could be important to reducing incidence of lameness in the industry (Caesar R et al, 2015; Ulluwishewa D et al, 2011). The wire flooring system is postulated to exert its effect from both stress and unstable

footing (Wideman RF, 2016, Wideman RF, et al 2012). We have also established that *S. agnetis* challenge can induce approximately 50% incidence of BCO on litter (Al-Rubaye AAK, et al, 2020) and that the bacterium can be spread through the air to neighboring pens. Birds grown on litter in separate pens but in the same room suffered lameness incidences of nearly 30%. We have tested for induction of lameness on wire flooring for four species isolated from lame birds: *S. agnetis, S. saprophyticus, S. epidermidis,* and *Enterococcus faecalis,* and one human isolate of *Staphylococcus aureus* (Al-Rubaye AAK, et al, 2015 -Al-Rubaye AAK, et al, 2016). Only the *S. agnetis* isolate induced lameness on wire and litter. In these same studies, we found that administering *S. saprophyticus* or *S. epidermidis* in the drinking water may actually reduce the incidence of lameness on wire-flooring. We have established that mixing birds that were challenged with *S. agnetis* with birds that were not challenged can increase the incidence of lameness in the non-challenged birds (Al-Rubaye AAK, et al, 2016). Our current working model is that *S. agnetis* can translocate across epithelial barriers, gain access to the blood, adhere to and infect the proximal growth plates of the long bones of the leg, and induce necrosis leading to lameness. *S. agnetis* may not be the primary cause of BCO lameness in commercial settings but it is an effective model for the spectrum of bacteria that are likely able to induce BCO lameness. Our experiments lead us to believe that *S. agnetis* has two alternative routes to translocate into the blood: the gastrointestinal route or the pulmonary route. We have identified prebiotics that can reduce lameness through the gastrointestinal route but do not protect against the pulmonary infection route. We hypothesize that administering *S. saprophyticu*s or *S. epidermidis* could reduce the incidence of BCO in birds after mixing with S. *agnetis* challenged birds on litter. We know these two species can reduce lameness in some conditions. This project will determine whether the reduction is primarily for the gastrointestinal or pulmonary routes, or both. Reducing

the incidence of lameness using Staphylococcus species can contribute to developing non-expensive effective probiotics to mitigate BCO loses in the poultry industry.

Materials and Methods

Cobb500 surplus males from a female broiler breeder were placed at 60 chicks per pen on litter for 24 pens (Table 1) and culled to 50 birds per pen at day 19 (Table 2). All birds were "walked" daily by being prompted with a broom. All pens received standard feed commercial chick starter (crumbles) from day 1-34 and broiler finisher (pellets) from day 35-56.

On days 20 and 21 the birds in pens 15-20 were administered *S. epidermidis*, pens 4-9 were administered *S. saprophyticus*, and pens 1-3, 13 & 14 were administered *S. agnetis*. To administer the three species of Staphylococcus, nipple waterers were switched from city water supply to 20 L carboys containing specific bacteria diluted in city tap water. All administrations were at 10^5 CFU/ml. On day 22, birds were returned to city tap water. From day 22 on cumulative lameness per pen was recorded with birds diagnosed as lame based on necropsy. On day 30, birds in pens 10, 21, 22, 15-17, & 4-6 were culled to 40 birds/pen. That same day, for those same pens, we added 10 birds from pens 1 & 13 which had been challenged with *S.* agnetis on days 20 and 21. The added birds were marked by green spray paint before mixing for easy identification. At the end of the experiment, day 56, we weighed and necropsied five seemingly healthy birds from each pen (a total of 115 birds) to determine the incidence of BCO in the proximal tibial and femoral for surviving, apparently healthy birds.

Bird densities initially were approximately 1.65 ft^2/chick in all pens. The project was performed in A365W at the University of Arkansas Poultry Research Farm. A365W is equipped with computer controllers to regulate the temperature, photoperiod, and ventilation. Tunnel ventilation and cool cells are automatically activated when needed. The photoperiod is set for 23 h light:1 h

dark for the duration of the experiment. At least two days before the chicks were placed, the waterers and carboys were flushed with diluted bleach (5 %), followed by a tap water flush, to remove bacterial biofilms. Thermoneutral temperature targets were set as follows: 90 °F for days 1 to 3, 88 °F for days 4 to 6, 85 °F for days 7 to10, 80 °F for days 11 to 14, and 75 °F thereafter. All broilers that die or that develop clinical lameness are recorded by date and pen, then are necropsied to assess BCO lesion distributions.

Clinical Diagnosis of Lameness

The birds were "walked" and observed for lameness every two days beginning on Day 15. Birds that are unwilling or unable to walk are diagnosed as "clinically lame" and humanely euthanized. All birds that die or develop clinical lameness were recorded by date, pen number, and if they had green paint. All suspected lame birds are necropsied and assigned to one of the following categories:

- Normal = Femur head and proximal tibia appear entirely normal
- Cull = Runts and individuals that failed to thrive or appeared to be clinically ill U = Unknown cause of death
- NE = Necrotic Enteritis
- SDS = Sudden Death Syndrome (Flip over, Heart Attacks)
- PHS = Pulmonary Hypertension Syndrome, Ascites
- KB = Kinky Back (Spondylolisthesis)
- TW = Twisted Leg or Slipped Tendon (perosis)
- TD = Tibial Dyschondroplasia
- Lame-UNK = Lameness for undetermined reasons
- FHS = Proximal Femoral Head Separation (epiphyseolysis)

- FHT = Proximal Femoral Head Transitional degeneration
- FHN = Proximal Femoral Head Necrosis (bacterial chondronecrosis with osteomyelitis, BCO) THN = Mild Proximal Tibial Head Necrosis, a sub-category of BCO in the tibiotarsus
- THNS = Proximal Tibial Head Necrosis Severe, THN in which the growth plate was imminently threatened or damaged
- THNC = Proximal Tibial Head Necrosis Caseous, THN in which caseous exudates or bacterial sequestrate were macroscopically evident

Lameness was calculated as follows:

- Total Lame = FHS + FHT + FHN + THN + THNS + THN
- Percent Lame = Total Lame/ (Total Birds - [sick or dead from other reasons])

Statistical Analyses

Data were statistically evaluated using a Generalized Linear Model (GLM) module in R.3.4.2 (https://cran.r-project.org/bin/windows/base/old/3.4.2/) to produce P-values between treatments. Significant difference was accepted at $P \leq 0.05$.

Results

This experiment was designed to determine whether birds raised on litter when in direct contact or simply rearing within the same room with *S. agnetis* challenged birds are susceptible to acquiring the infection inducing lameness. We also wanted to determine whether exposure to non-pathogenic Staphylococci could protect against the infection spread, whether through direct contact or airborne transmission. In Figure 9 we present the cumulative lameness for each of the treatments. Note that there were different numbers of birds in different treatment groups. There

were three pens per treatment group, but there were 50 birds per pen in the unmixed, 40 in the "target" group in mixed pens, and 10 in the "source" group in the mixed pens. We did not see significant variation between triplicate pens in the different treatments (Table 3). GLM analysis of the final lameness values showed that the only significant difference was for the "source" birds, which represent the *S. agnetis* challenged birds that were mixed with birds with no challenge, or birds treated with *S. epidermidis* or *S. saprophyticus* (green lines with markers Figure 9; Table 4). Those birds were only significantly different from the *S. epidermidis* challenged birds that were not mixed (S.ep_NoMix). It is also important to note that the Source birds were 10 birds per pen rather than 40 for the Target, or 50 for the NoMix. There were no apparent differences between treatments in the severity of BCO lesions that were diagnosed at necropsy (Figure 10). Body weights for apparently normal healthy birds at the end of the experiment showed no significant difference between body weights for any of the seven treatment groups: Target and NoMix (Table 4). Note: we did not evaluate body weights for the "source" birds used for mixing because there were only a few that survived to the end of the experiment.

Discussion

This experiment was designed to follow-up findings from several of our previous experiments. In earlier experiments when we treated birds raised on wire with the three species of Staphylococcus used here, the *S. agnetis* challenged birds had a higher incidence of lameness than unchallenged birds, and the *S. epidermidis* or *S. saprophyticus* challenged birds had lameness incidences that were less than the unchallenged birds (Alrubaye AAK, et al, 2016). This led us to hypothesize that these "non-pathogens" could induce some form of resistance

through either competitive exclusion or enhanced immune function(s). It is important to note that the reduced incidence of lameness in those experiments was under conditions where the different treatments were in separate chambers in PERL, not in separate pens in a single room, and the birds were raised on wire flooring. Early this year we tried challenging birds raised on litter with *S. agnetis*. The incidence of lameness was 50% and for unchallenged birds in separate pens in the same room the incidence of lameness was 30%. We had earlier found that challenged birds could induce equivalent levels of lameness in unchallenged birds when the birds were mixed, but this was again an experiment on wire flooring. Of significant concern is that the final incidence of lameness in this latest experiment is around 70-80% which is roughly the highest level of lameness we have ever seen for *S. agnetis* challenged birds raised on wire flooring so now we have attained much higher incidence of lameness than we had seen in Experiment 21 for challenged birds raised on litter. We have to ask why the level of lameness was so high when we observed lower levels in the previous experiment. We speculate that there are two possibilities: 1) there was insufficient cleaning and sanitization of the facility between the last experiment and this experiment so that there was a heavy environmental load of *S. agnetis* in the room, or 2) the previous experiment was in May and June whereas this experiment completed in mid-November. That would mean that the facility needed less air movement, and the exhaust fans would not have run continuously. This would have led to a much higher level of contaminated litter dust in the air which may have meant a higher burden of suspended particles coated with *S. agnetis* for this latest experiment. We also noted one other significant deviation in this latest experiment. Previously for experiments using birds raised on wire flooring we had found that a large percentage of lame birds were positive for viable *S. agnetis* in their blood. When we performed microbiological sampling of blood for either *S. epidermidis* or *S. agnetis* challenged birds (n=20)

we only found a few birds that were positive. We also evaluated blood from apparently healthy birds at the end of the experiment and found only 1 of 20 were positive. Thus, the actual numbers of viable bacteria in blood in this latest experiment using birds on litter was lower than for birds on wire. In Experiment 17 we had also found that the bacteremia was lower in birds on litter than on wire (Alrubaye AAK, et al, 2016), but that was in the absence of the challenge with *S. agnetis* in the drinking water. In experiment 17 the birds on litter had $1/10^{th}$ the number of viable bacteria in blood whether we assessed healthy or lame birds. There is clearly more about the environmental and physiological aspects of BCO lameness that we need to discern.

One significant observation derived from Experiment 22 is the distribution of particular BCO diagnoses between experiments using wire and litter flooring. The initial observation was that there were few FHN diagnoses for the lame birds raised on litter. The BCO diagnoses for tibiae and femorae for birds diagnosed as lame from Experiment 19 on wire flooring, and Experiment 22 on litter flooring are presented in Figure 12. Experiment 19 involved a similar challenge with *S. agnetis* where challenged birds were then mixed with unchallenged birds but Experiment 19 was for birds raised on wire flooring. The incidence of lameness for Experiment 19 was around 70-80% for challenged birds, and also for unchallenged birds that were mixed with challenged birds. So, the incidence of lameness was comparable for Experiment 19 and Experiment 22. Examination of the BCO diagnoses for tibiae show that nearly all the birds had severe BCO in both right and left tibiae in both experiment 19 and 22 (Figure 11). However, we noted a distinction between Experiment 19 and 22 for the BCO diagnoses for BCO of the femoral. For Experiment 19 the distribution was fairly uniform (20-25%) for normal and all three categories of femoral head BCO (FHS, FHT, and FHN), but for Experiment 22 there were few cases of normal or Femoral Head Necrosis (FHN), with almost all diagnoses equally distributed between

Femoral Head Separation (FHS), and Femoral Head Transitional (FHT). Thus, for the birds on litter the lameness diagnoses were almost all in the mid-range of severity, with very few diagnosed as normal. Figure 12 presents the BCO diagnoses from Experiment 17 where we raised birds on litter (L56), on wire (W56), or on litter for 35 days then on wire through day 56 (L35W). For this experiment, there was no administration of bacterial challenge and so the source of the infection was entirely from the environment. Cumulative lameness was 3% for L56, 53% for L35W, and 71% for W56 (Alrubaye AAK, et al, 2016). We can see from Figure 12 that the L56 lame birds had similar tibial diagnoses, as for the other Experiment 17 treatments and for Experiment 19 and 22 (Figure 10 and Figure 11). However, the L56 had less severe femoral diagnoses than did the L35W or W56, much the same as the femoral diagnoses for Experiment 22 (Figure 10 and Figure 11). It may be that the lower incidence of bacteremia is related to the reduced severity of femoral BCO progression. We have always believed that the bacteremia is a result of the severe necrosis resulting from osteoclast degradation of the infected bone. If the majority of the femoral BCO is Femoral Head Separation (FHS) then it may be that the infection is still restricted to the growth plate. Future experiments should be designed to examine lameness with a lower quantity of S. agnetis in the drinking water. Our next experiment will use 104 CFU/ml rather than 105. Which was about half the incidence of lameness for birds raised on wire (Alrubaye AAK, et al, 2016). Also, try continuous application of non-pathogenic Staphylococci in the drinking water to see if that can protect better than a single dose. Try direct tracheal application of the bacterial challenge. We could then monitor blood for how soon we can detect viable bacteria. Set up a better airflow system to reduce spread of the bacteria in the aerosol. It would be important to do daily microbial monitoring for S. agnetis in the air to assess

microbial load. Finally, we need to do a more extensive survey for blood bacteremia for birds challenged with S. agnetis raised on litter.

Table 1. Pen Setup for this project

Treatment	Description	
A	No Challenge No Mix (Pens 11,12,23)	Start 50 supplement, if necessary, from pen 24 through day 19
B	No Challenge Mix with Challenged birds' day 30 (Pens 10,21,22)	Start 50 birds; day 30 cull to 40 then add 10 birds from treatment H
C	*S. epidermidis* day 20 No Mix (Pens 18,19,20)	Start 50 supplement, if necessary, from pen 24 through day 19
D	*S. epidermidis* day 20 Mix with Challenged birds' day 30 (Pens 15,16,17)	Start 50 supplement, if necessary, from pen 24 through day 19
E	*S. saprophyticus* day 20 No Mix (Pens 7,8,9)	Start 50 supplement, if necessary, from pen 24 through day 19
F	*S. saprophyticus* day 20 Mix with Challenged birds' day 30 (Pens 4,5,6)	Start 50 birds; day 30 cull to 40 then add 10 birds from treatment H
G	*S. agnetis* challenge on day 20 no mix (Pens 2,3,14)	Start 50 supplement, if necessary, from pen 24 through day 19
H	*S. agnetis* challenge on day 20 use for mixing (Pens 1,13)	Start 50 supplement, if necessary, from pen 24 through day 19

Table 2. Protocol for this project

Age	Date	Day	Temp. °F	Comments
1	26-Sep	Monday		Place 50 chicks on litter flooring. Pens 1-24 Cobb Starter.
19	14-Oct	Friday		Supplement any pens to 50 from pen 24
20	15-Oct	Saturday		• Pens 15-20 will be administered *S. epidermidis* • Pens 4-9 will be administered *S. saprophyticus*, and • Pens 1-3, 13 & 14 will be administered *S. agnetis* All at 10^5 cfu/ml in drinking water for 2 days.
21	16-Oct	Sunday		
22	17-Oct	Monday		Begin recording all deaths, lame and infirmed.
30	25-Oct	Tuesday		• Birds in pens 4-6, 10, 15-17, 21 and 22 will be culled to 40 bird/pen. Ten birds from pens 1 & 13 will be added into pens 4-6, 10, 15-17, 21 and 22.
35	30-Oct	Sunday		Switch all pens to Cobb finisher.
56	20-Nov	Sunday		Complete experiment. Weigh and necropsy 5 apparently healthy birds from each of the 7 treatment groups (35 birds total).

Table 3. Final Percent Lameness for triplicate pens for the different treatment groups. For the pens challenged with anything other than S. agnetis, there were three pens which were not mixed and three pens which were mixed with 10 birds challenged with S. agnetis

Challenge	Mix	N per pen	Percent Lameness		
			pen1	pen2	pen3
None	No	50	77	76	88
None	Yes	40	79	78	85
S. epidermidis	No	50	78	70	80
S. epidermidis	Yes	40	53	95	95
S. saprophyticus	No	50	92	62	66
S. saprophyticus	Yes	40	83	65	63
S. agnetis	No	50	76	66	78

Table 4. GLM analyses of statistical significance for different treatment groups. The groups are as in Table 1 and the text. Treatments where there was significant difference (P<0.05) the P-value is in red text.

	Challenge	none	none	S.ep	S.ep	S.sap	S.sap	S.agnetis	S.agnetis	S.agnetis	S.agnetis
Challenge		NoMix	Target	NoMix	Target	NoMix	Target	None_Source	S.ep_Source	S.sap_Source	NoMix
none	NoMix		0.666	0.111	0.663	0.658	0.606	0.141	0.124	0.170	0.980
none	Target	0.666		0.263	0.997	0.993	0.905	0.101	0.089	0.127	0.708
S.ep	NoMix	0.111	0.263		0.262	0.350	0.482	0.035	0.031	0.042	0.221
S.ep	Target	0.663	0.997	0.262		0.989	0.898	0.101	0.089	0.121	0.699
S.sap	NoMix	0.658	0.993	0.350	0.989		0.873	0.105	0.091	0.087	0.583
S.sap	Target	0.606	0.905	0.482	0.898	0.873		0.102	0.089	0.074	0.485
S.agnetis	None_Source	0.141	0.101	0.035	0.101	0.105	0.102		0.965	0.764	0.160
S.agnetis	S.ep_Source	0.124	0.089	0.031	0.089	0.091	0.089	0.965		0.726	0.141
S.agnetis	S.sap_Source	0.170	0.127	0.042	0.121	0.087	0.074	0.764	0.726		0.144
S.agnetis	NoMix	0.980	0.708	0.221	0.699	0.583	0.485	0.159	0.140	0.144	

Table 5. Body weights for apparently healthy birds on day 56 at the end of the experiment. Five birds from each treatment were weighed, euthanized and evaluated for BCO lesions. The average weights and standard deviations are presented as well as P-values from T-test for comparisons between treatments

Challenge	Mix	Treatment	avg weight	std	T-Test P value					
					B	C	D	E	F	G
none	no	A	4.074	0.35	0.318	0.445	0.162	0.106	0.176	0.070
none	yes	B	4.164	0.21		0.295	0.232	0.109	0.250	0.084
S. epidermidis	no	C	4.038	0.45			0.169	0.127	0.171	0.086
S. epidermidis	yes	D	4.272	0.23				0.385	0.422	0.230
S. saprophyticus	no	E	4.306	0.08					0.480	0.254
S. saprophyticus	yes	F	4.316	0.42						0.338
S. agnetis	no	G	4.394	0.26						

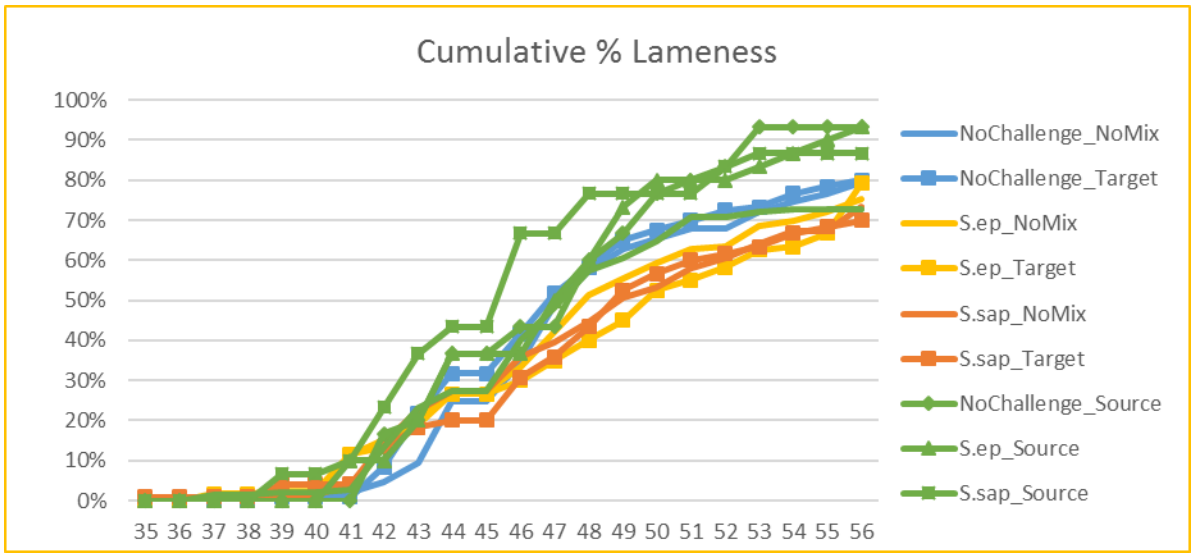


Figure 9. Cumulative lameness for the 7 treatment groups. The treatments are coded by color for the treatment: green challenged with S. agnetis; blue- no challenge; orange- challenged with S. epidermidis; red- challenged with S. saprophyticus. Markers indicate mixing: no marker- no mixing; square- mixed with S. agnetis challenged birds. Target are birds untreated or challenged with non agnetis that were mixed with S. agnetis challenged birds. Source birds were those challenged with S. agnetis and then mixed with untreated or challenged with non-agnetis challenged birds.

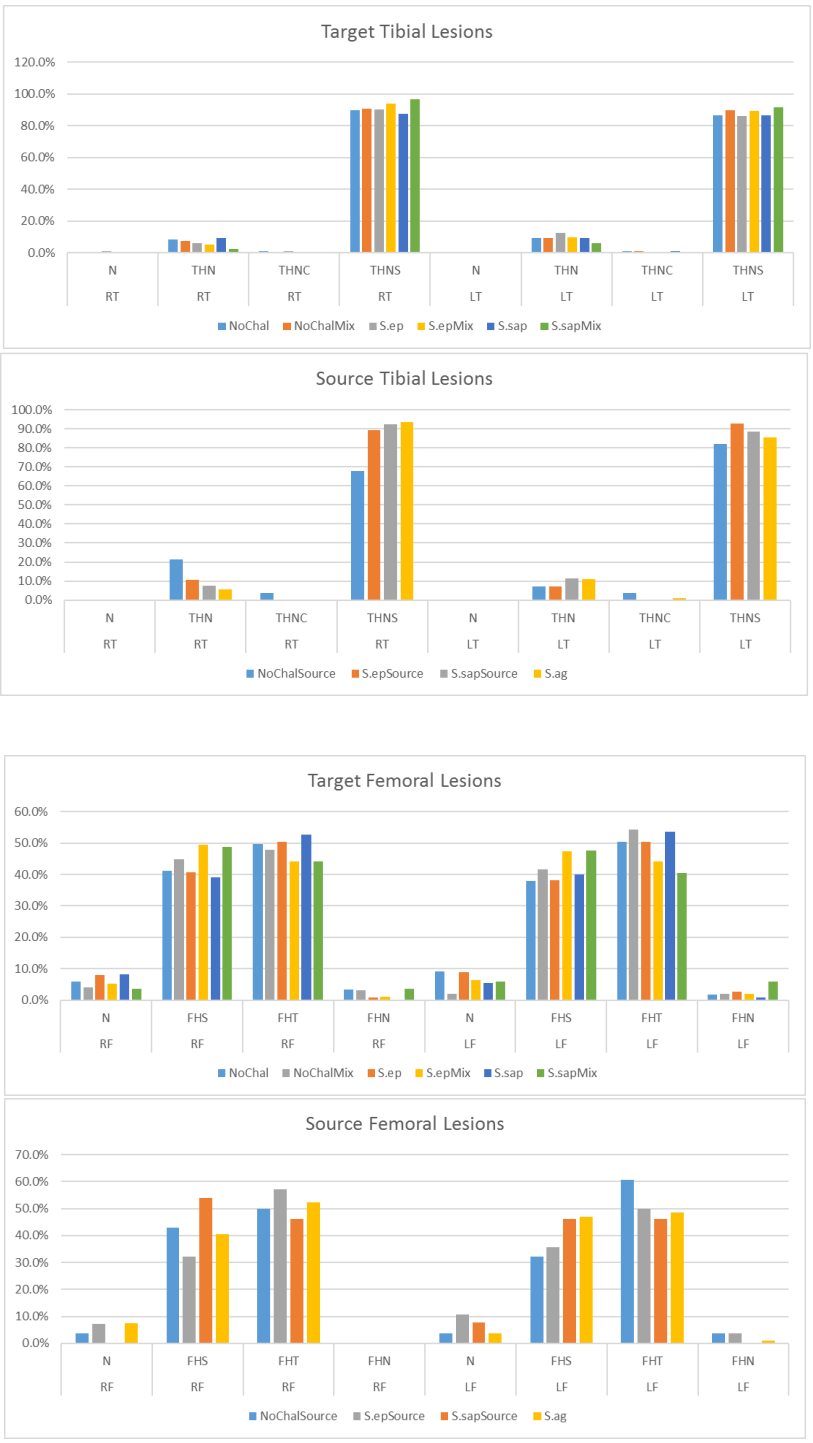


Figure 10. Lesion diagnoses for Tibiae and Femoral for Target or Source treatments (see text). BCO severities by percent of lame birds are plotted for Right Tibia (RT), Left Tibia (LT), Right Femoral (RF) and Left Femoral (LF). Abbreviations for BCO severity are as presented in the text. Abbreviations for Source and Target are as in Figure 10.

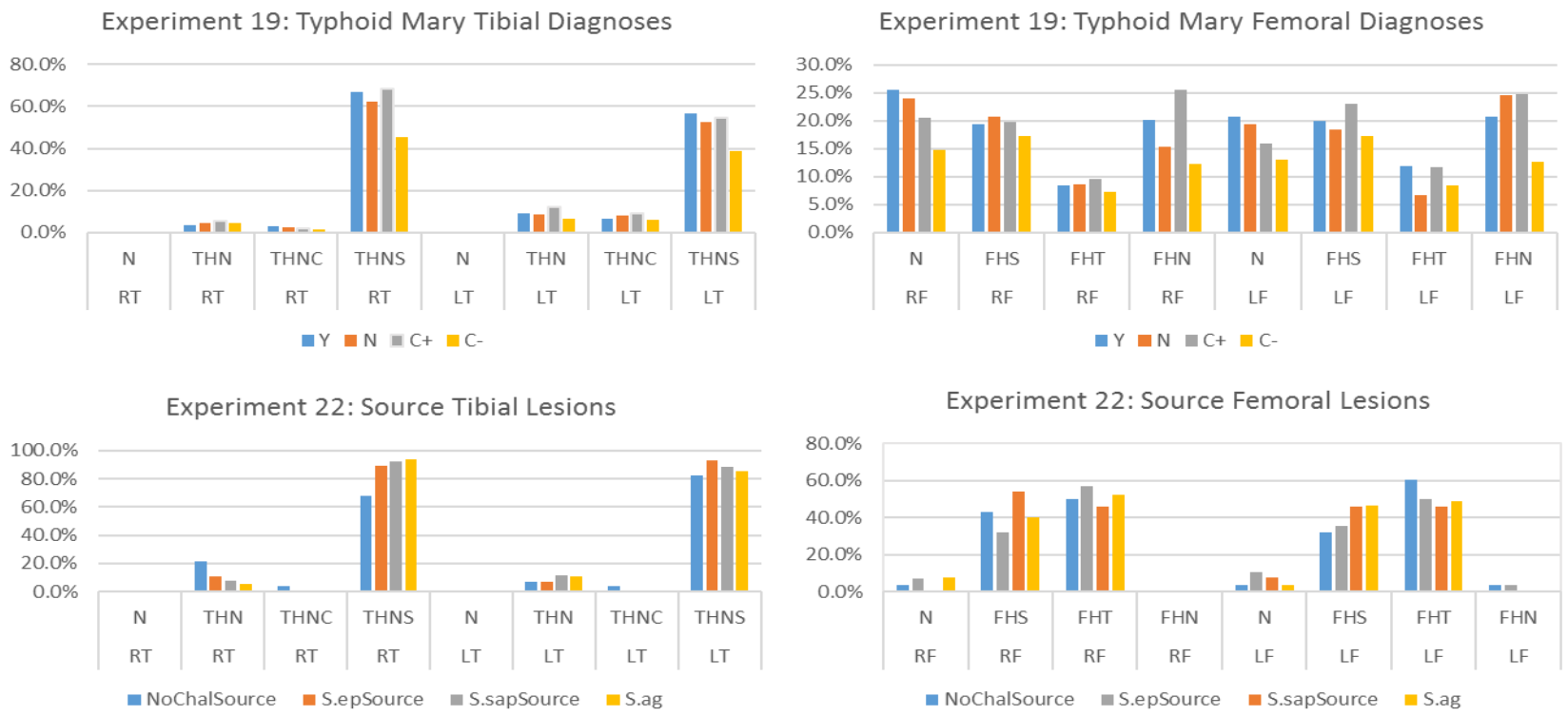
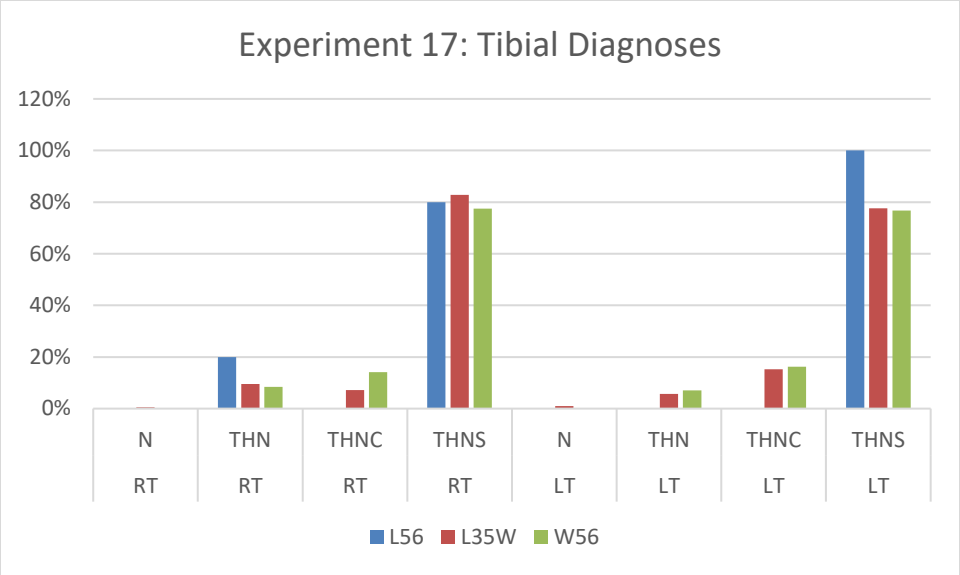


Figure 11. Comparison of tibiae (left panels) and femoral (right panels) BCO diagnoses between birds on wire (Experiment 19) vs litter (Experiment 22). Experiment 19 involved a bacterial challenge to birds raised on wire where C+ and Y represent birds challenged with S. agnetis, and C- and N were birds not challenged. The Y and N birds were then mixed 50:50 at day 30. The incidence of lameness was statistically the same for C+, Y and N at around 70-75% with the incidence of lameness in C- being lower (statistically significant) at 50%. BCO diagnoses are as in Figure 5 and the text. Experiment 22 figures are reproduced from Figure 11.



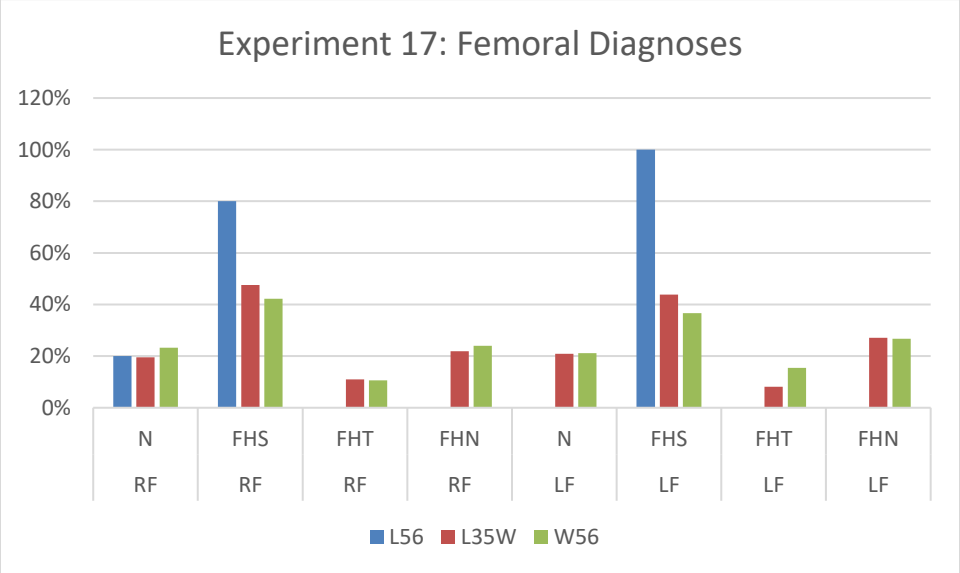


Figure 12. Experiment 17 BCO diagnoses for lame birds comparing BCO diagnoses for birds on litter for 56 days (L56), Litter for 35 days and then 21 days on wire (L35W), and wire for 56 days (W56). Tibial and Femoral diagnoses are plotted as percent of lame birds with BCO and bone designations as in the text and Figure 11.

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CHAPTER 3

Chondronecrosis with osteomyelitis in broilers: further defining a bacterial challenge model using standard litter flooring and protection with probiotics

Chondronecrosis with osteomyelitis in broilers: further defining a bacterial challenge model using standard litter flooring and protection with probiotics

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Chondronecrosis with osteomyelitis in broilers: further defining a bacterial challenge model using standard litter flooring and protection with probiotics

Abstract

This report demonstrates that high levels of lameness can be induced by a limited bacterial challenge in drinking water for birds raised on litter flooring, comparable with lameness induced by the gold standard for inducing lameness, growth on suspended wire flooring. The bacterium used in the challenge was cultured from lesions in birds induced for bacterial chondronecrosis with osteomyelitis (BCO) in the wire-flooring model, so the epidemiology appears similar. The litter-flooring model could better approximate broiler operations. Furthermore, the work demonstrates that 2 commercial probiotics (GalliProTect and GalliProMax) can reduce lameness in the bacterial challenge litter-flooring model. Lameness attributable to BCO is one of the most significant animal welfare issues for broiler production. The wire-flooring and litter-flooring models afford alternatives for understanding the etiology, and epidemiology of BCO, and development of management strategies to reduce lameness. Probiotics afford a promising management strategy. The results suggest that the probiotic protection may extend beyond just intestinal health and intestinal barrier function.

Introduction

Lameness is one of the most significant animal welfare issues in the broiler industry, resulting in annual losses of millions of dollars. A wire-flooring model has been shown to induce high incidence of lameness in broilers (Wideman et al., 2012; Wideman and Prisby, 2013; Wideman et al., 2013; Wideman et al., 2014; Wideman, 2016). Bacterial chondronecrosis with osteomyelitis (BCO) of the proximal tibiae and femora is the most prevalent form of lameness in this model (Wideman et al., 2012; Wideman and Prisby, 2013; Wideman et al., 2013; Wideman, 2016). The wire-flooring system led to a model for BCO susceptibility based on the vasculature and growth plate dynamics (Wideman and Prisby, 2013; Wideman, 2016). The predominant isolates from BCO lesions using the wire floor model on our research farm is Staphylococcus agnetis and BCO lameness is sometimes associated with a significant bacteremia (Al-Rubaye et al., 2015). The isolates of S. agnetis appear to be from a clonal population, and administration of the type of strain, isolate 908, in the drinking water increases the incidence of lameness (Al-Rubaye et al., 2015). Further investigations on the BCO lameness model demonstrated translocation of bacteria into the blood for birds on litter or wire flooring; and evidence for transmission of BCO-inducing pathogens within a flock (Al-Rubaye et al., 2017). We now report further investigations on, and expansion of, the BCO lameness model system. We now report that S. agnetis 908 can induce high levels of BCO lameness in birds raised on litter flooring. We also report that BCO lameness can be transmitted through the air to broilers in separate pens. Furthermore, broilers fed either of 2 commercial probiotics are protected against the airborne spread of the infection. These data provide a model system for studying treatments and management strategies for reducing BCO lameness in broiler operations.

Materials and methods

Lameness Trials

All animal experiments were approved by the University of Arkansas Institutional Animal Care and Use Committee under protocols 15043, 16073, 16073-1, and 17067. One-day-old chicks representing surplus males from a female broiler-breeder product were placed in 1.5 × 3 m pens on fresh wood shaving litter at 60 per pen, with a nipple water line supplied with city tap water on one 5 ft. side and 2 feeders on the other side.

Feed was standard starter through day 35 and finisher through day 56. For administration of probiotics, the commercial product (Table 6) was added by the University of Arkansas Poultry Research Feed mill to standard feed formula (Supplementary Table 10) before pelleting. Lines and mixers were flushed and cleaned between formulations. Samples of the pelleted feed were shipped to the supplier for verification of probiotic viability. For these experiments, we had up to 4 treatment groups as described in Table 10. In all experiments, there was a source population that was challenged with S. agnetis 908 in the drinking water, and a control population that was not challenged but was housed in the same room “down wind” of the source population. Other treatments were the same as the control but with administration of probiotics in the feed. Treatment pens were arrayed using a randomized block design.

Computer controllers regulated the temperature, photoperiod, and ventilation. Tunnel ventilation and evaporative cool pads were automatically activated when needed. The photoperiod was set for 23 h light:1 h dark for the duration of the experiment. Thermoneutral

temperature targets were as follows: 90°F for day 1 to day 3, 88°F for day 4 to day 6, 85°F for day 7 to day 10, 80°F for day 11 to day 14, and 75°F thereafter. On day 19, all pens were culled to 50 birds. Pens challenged with S. agnetis were located "upwind" from all the other pens so that the ventilation flow would be from the challenged pens (source) to the downwind treatment pens (control or probiotic). For pens challenged with S. agnetis in the drinking water, at 7 AM on day 20, the hose to the nipple waterers was attached to an elevated 20L carboy. The bacteria (stationary overnight culture) were mixed into tap water in the carboy to 105 CFU/mL. At 7 PM on day 21, the nipple supply was restored to the tap water. All water lines were flushed with dilute bleach and fresh tap water before any experiment. Beginning with day 20, all birds were encouraged twice per day to move using standard kitchen brooms. Any bird that was reticent to move was marked with spray paint. Birds that were unwilling or unable to walk were diagnosed as "clinically lame" and euthanized. All birds that died or were diagnosed as clinical lame were recorded by date and pen number. Necropsy for BCO lameness was as described (Wideman, 2016) to assign as either N = normal proximal femur head or proximal tibia head; KB = kinky back (spondylolisthesis); FHS = proximal femoral head separation (epiphyseolysis); FHT = proximal femoral head transitional degeneration; FHN = proximal femoral head necrosis; THN = proximal tibial head necrosis; or other = symptoms other than BCO. Total BCO lame included all birds diagnosed as having FHS, FHT, FHN, THN, or KB. Some lame birds were also sampled for bacterial species in blood and BCO lesions as described (Al-Rubaye et al., 2015; Al-Rubaye et al., 2017). Air sampling used open petri dishes of ChromAgar Orientation (DRG International, Springfield, NJ). Species identification included subculture on ChromAgar Orientation, and ChromAgar

Staphylococcus (DRG International) followed by PCR sequencing of 16S rRNA (Al-Rubaye et al., 2015; Al-Rubaye et al., 2017).

Transepithelial Electrical Resistance and Short-Circuit Current

Ileal transepithelial electrical resistance (TEER) and short-circuit current (SCC) was from 10 apparently healthy birds from each treatment on day 57. A 5-cm section of proximal ileum (1 cm distal to Meckel's diverticulum) was excised from freshly euthanized birds, rinsed with cold 1× phosphate-buffered saline, and were transported in fresh Kreb's buffer (Koltes et al., 2017) on ice to the main campus (about 5 min), and analyzed immediately. The TEER and SCC were measured in the Kreb's solution in an 8 chamber Navicyte Vertical EasyMount Ussing system (Warner Instruments, Hamden, CT) (Koltes et al., 2017). Data were captured in Acquire & Analyze revII (Physiologic Instruments, San Diego, CA) over a 20-min period. When all sample TEER and SCC were stable, an average of 5 min were used for the final data analyses.

Histopathology Analyses

Intestinal samples (3 cm section) for histopathology were the distal jejunum (1 cm proximal to Meckel's diverticulum) and the proximal ileum (1 cm distal to Meckel's diverticulum). Sections were collected on day 57 from euthanized apparently healthy birds. Samples were flushed with 1× phosphate-buffered saline and fixed for 1 to 2 d in phosphate-buffered 10% formalin (Cat# JTM518-3, J.T. Baker). The fixed samples were processed through the histology laboratory in the Department of Poultry Science at the University of Arkansas.

Hematoxylin–Eosin-stained sections were imaged on an Olympus inverted scope at 400× using a CCD camper to display on an LCD monitor. Villus length was measured on the monitor screen with a flexible ruler. Calibration was based on a stage micrometer. For villus length and pathology, at least 4 sections were examined for each tissue for each bird (8–10 birds per treatment). For each section, villus length was measured, and gross pathology (villus tip integrity) was scored, on 4 sides (top, left, right, and bottom). For some sections, villus length and tip integrity could not be measured on all 4 sides owing to tissue damage in sectioning.

Statistical Analyses

Data were statistically evaluated using either the T-test function in Microsoft Excel (Office 365, Microsoft Corp., Redmond, WA) or a generalized linear model module in R.3.4.2 (https://cran.r-project.org/bin/windows/base/old/3.4.2/) to produce P-values between treatments. Significant difference was accepted at $P \leq 0.05$.

Results and discussion

In our previous publications, we had reported that S. agnetis 908 can induce lameness at 80 to 90% for birds raised on wire flooring (Al-Rubaye et al., 2015; Al-Rubaye et al., 2017). This work also demonstrated that S. agnetis 908 can be transmitted from challenged birds to pen mates for birds raised on wire flooring. From that work, we hypothesized that contact or aerosols could spread the infection. Therefore, for experiment 1, we tested whether S. agnetis 908 administrations could induce BCO lameness for broilers raised on standard litter flooring. We also tested whether the BCO infection could be transmitted to broilers in the same environment but not in direct contact with the challenged birds. For experiment 1, we had 14

pens of 50 birds in each of 2 treatment groups (n = 700 birds per treatment), source and control (Table 6). Source received a challenge with S. agnetis 908 in drinking water for day 20 and day 21 (Methods), whereas control received no challenge but was located within the same room. Cumulative lameness through day 56 shows that birds raised on litter and administered S. agnetis 908 at 105 CFU/mL in drinking water for day 20 and day 21 experienced cumulative lameness at 47% (Figure 13). The unchallenged control birds in the separated pens experienced a cumulative lameness of 31%, and the appearance of lameness in the control birds was delayed by about 5 d relative to the challenged source birds. The average counts per pen of lame birds in the source treatment was 23.4 ± 0.7 (SEM) and in the control pens lame bird counts was 15.4 ± 1.0 (T-test P-value = 1.5 × 10−6). Although we had no negative control population in a separate facility, typical lameness values for broiler breeders on litter flooring are usually less than 5% (Wideman et al., 2012; Wideman et al., 2015; Wideman, 2016), except in cases of lameness outbreaks (personal communications from industry sources). The severity of the BCO lesions in lame birds was very similar for the source birds and the control birds (Figure 14). Microbiological sampling of blood and BCO lesions from the control birds exclusively recovered S. agnetis. Therefore, the bacteria had spread from the source to the control birds, as we had found for birds raised on wire flooring (Al-Rubaye et al., 2015; Al-Rubaye et al., 2017). In addition, we sampled the air within the building using 10 open petri plates of CHROMAgar Orientation. We recovered thousands of pink colonies that and primarily recovered Staphylococcus saprophyticus but approximately 3% of the colonies were S. agnetis. Therefore, the bacterium in the source birds was being aerosolized within the facility.

Previously, we had tested probiotics and gut health supplements for protection against BCO lameness for birds raised on wire flooring (Wideman et al., 2012; Wideman et al., 2015; Al-Rubaye et al., 2017). Specifically, we had reported that Biomin PoultryStar or Quality Technology International Incorporated BacPack 2X (combined prebiotic and probiotic) can reduce incidence of lameness by approximately 50% for birds raised on wire flooring (Wideman et al., 2012; Wideman et al., 2015). However, we later reported that when we challenged broilers with S. agnetis 908 in the drinking water at 20 d of age the protective effect of PoultryStar was overwhelmed (Al-Rubaye et al., 2017). Therefore, we designed experiment 2 to examine the activity of 2 commercial probiotics, GalliProTect and GalliProMax, for protective effect against the transmission of S. agnetis 908 induced BCO lameness from a challenged source treatment (Table 6). There were 4 pens of 50 birds each in the source (challenged with S. agnetis 908 on normal feed), and the control (normal feed). There were 5 pens each for the 2 probiotic products. The experiment was set up with the source pens "upwind" of the other pens relative to the exhaust fans. The control, GalliProTect, and GalliProMax pens were arrayed in a randomized, block design, and separated from the source pens by empty pens. Figure 15 shows the cumulative percentage lameness for the 4 treatments through day 56. Incidence of lameness in the source population was higher than in experiment 1 (77% vs. 56%). Experiment 1 was conducted from April 14 through June 8, 2016, whereas experiment 2 was March 8 through May 3, 2017, so outside temperatures (data from NOAA website) were milder during experiment 2 and there were no unusual heat stresses. The higher incidence of lameness in experiment 2 may reflect increasing contamination of the facility with S. agnetis 908 through repeated experiments similar to the increasing lameness with continued experiments on growth on wire flooring (Al-Rubaye et

al., 2017). However, the facility is sprayed down with dilute bleach and power washed between experiments. The lameness incidence in the control and probiotic treatments were lower than the source pens and only during the last 4 d was there separation between the control treatment and the probiotic treatments, GalliProTect and GalliProMax, which were equivalent in their protective effect. The pen-to-pen variation in lame birds was similar within a treatment and non-lameness associated mortality was low (Table 7). P values for comparisons of the 4 treatments (Table 8) confirm that lameness in the source treatment was higher than the 3 other treatments, and that the 2 probiotic treatments were lower than the control treatment ($P \leq 0.02$; Table 8). Lameness relative to the control was reduced by 25% by GalliProTect and 20% by GalliProMax, but the 2 probiotics were not significantly different from each other ($P = 0.41$; Table 8). The distribution of BCO lesion severity was not substantively different between the 4 treatments (Figure 16) except for reduced femoral lesion severity in the GalliprotTect for the left, but not the right, femoral head. We suspect this is an experimental aberration as we have reported left/right variability before (Wideman et al., 2012; Wideman et al., 2013; Gilley et al., 2014; Wideman, 2016; Al-Rubaye et al., 2017). We weighed 8 apparently healthy birds randomly selected from each of the treatments on day 56 and observed that the source birds were slightly heavier, at least relative to the control and GalliProMax (Table 11). Whether this difference was because the extreme disease pressure in the source treatment selected for highly robust birds, or was a statistical artifact, is not known without further investigation.

We collected intestinal tissue from 10 apparently healthy birds on day 57 (held over from the end of the experiment) from the control, GalliProTect, and GalliProMax treatments. The

proximal ileum was analyzed for permeability by TEER and SCC in Ussing chambers. The SCC was not different across treatments (P-value = 0.135). Average SCC for control was −16.08 ± 1.82; GalliProText was −11.35 ± 1.54; and GalliProMax was −14.43 ± 1.35. The TEER was significantly higher in GalliProTect and increased but not at the level of significance in GalliProMax (Figure 17). Histopathology was conducted on paired samples of the ileum and jejunum from the same birds for ileal TEER and SSC. Regions of the ileum and jejunum were also assessed for villus length and gross pathology (Materials and Methods). Villus length data are presented in Table 10. Ileal villus length was significantly higher (P-value < 0.001) in both probiotic treatments when compared with the control group. Villus length in the jejunum was lower than control for the GalliProTect but higher than control for GalliProMax. We also scored the jejunum for disrupted villus tips (Table 6). The control treatment had a higher number of birds with ruptured villus tips and 50% of the sections had evidence of ruptured villus tips. GalliProMax had the fewest birds with ruptured villus tips and the lowest percentage of sections with ruptured villus tips.

These experiments were designed to extend our development of models for investigation of the epidemiology and etiology of BCO lameness in broilers. The wire-flooring model provides a method for inducing lameness and established that BCO was the primary form of lameness induced on wire flooring. Those investigations yielded a strain of S. agnetis that is the primary isolate from lame broilers in our facilities when birds are raised on wire flooring. We now extend the model using this S. agnetis isolate to demonstrate that this culture can induce significant levels of lameness in broilers raised on standard litter floors. This new litter floor model of BCO lameness recapitulates the lameness outbreaks that can occur in particular

broiler houses where lameness is recorded in one area of the building and then spreads through the building. As was found with the wire-flooring model for lameness, some commercial probiotics can reduce the incidence or delay the onset of lameness. Probiotics are theorized to improve gut health and reduce pathogen colonization of the gut. However, even though we administer S. agnetis 908 in the drinking water to the source birds, the most likely transmission to the control, and probiotic treated birds is through aerosols. Therefore, the most likely route of the infection in those birds is the pulmonary route. An alternative is that the aerosolized bacteria contaminate feed or waterers in the other pens and is consumed rather than inhaled.

Conclusions

We were the first to report S. agnetis infections in poultry (Al-Rubaye et al., 2015), and there are few other reports of S. agnetis isolation from poultry (Poulsen et al., 2017). Before these reports, S. agnetis had been first identified in isolations from mastitis in cattle (Taponen et al., 2012; Calcutt et al., 2014; Adkins et al., 2017). Phylogenomic analysis of all known S. agnetis genomes has revealed that the poultry isolates may have radiated from a clade within the cattle isolates (Shwani et al., 2020). These analyses have failed to identify chicken-specific genetic determinants. In recent surveys of BCO lameness outbreaks in Arkansas broiler operations, we have isolated Staphylococcus aureus, Staphylococcus hyicus, Enterococcus cecorum, and Escherichia coli (Ekesi et al., 2021). Similar species of bacteria have been associated with BCO in other studies (Carnaghan, 1966; Devriese et al., 1972; Emslie and Nade, 1983; Kibenge et al., 1983; Mutalib et al., 1983; Griffiths et al., 1984; Julian, 1985; Daum et al., 1990; Duff, 1990; Hocking, 1992; Butterworth 1999; Butterworth et al., 2001;

Ytrehus et al., 2007; Kense and Landman, 2011; Borst et al., 2015; Alstrup et al., 2016; Marek et al., 2016; Wideman, 2016). Whether these other BCO isolates are as virulent as S. agnetis 908 is not known, nor do we know whether they can be transmitted as readily as S. agnetis 908 under standard broiler rearing conditions. We have speculated that S. agnetis 908 evolved into a hypervirulent strain during the years of inducing BCO lameness in our research facilities. However, similar selection in broiler grower facilities can also be going on in each broiler operation. Each broiler operation could be evolving its own particular strain. This is consistent with anecdotal evidence from broiler operators that particular broiler houses have continuing issues with lameness incidence. The litter-flooring model for BCO lameness clearly demonstrates that the BCO epidemic can spread through the air for our S. agnetis model, and this may be likely for the other BCO-associated bacterial species. We have now demonstrated that that probiotic can reduce the spread of the infection through the air. If the primary spread of the infection is through the pulmonary route, then either the probiotics are also being introduced to the pulmonary system or the probiotic bacteria are eliciting an enhanced generalized innate immunity in major epithelial layers.

The wire-flooring model for inducing lameness and the litter-flooring model for BCO lameness epidemics, provide avenues to develop strategies and evaluate management programs to reduce a continuing animal welfare concern.

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Appendix

Table 6. Treatment designations and descriptions for treatments in experiments 1 and 2.

Treatment	Description
Source	Control diet challenge with *Staphylococcus agnetis* 908 10^5 CFU/mL in drinking water on days 20&21
Control	Control diet
GalliProTect	GalliPro Tect diet 3.2 × 10^9 CFU *Bacillus licheniformis* DSM17236/g of product at 0.5 kg/metric ton of feed
GalliProMax	GalliPro Max diet 1.6 × 10^9 CFU *Bacillus subtilis* DSM17299/g of product at 0.5 kg/metric ton of feed

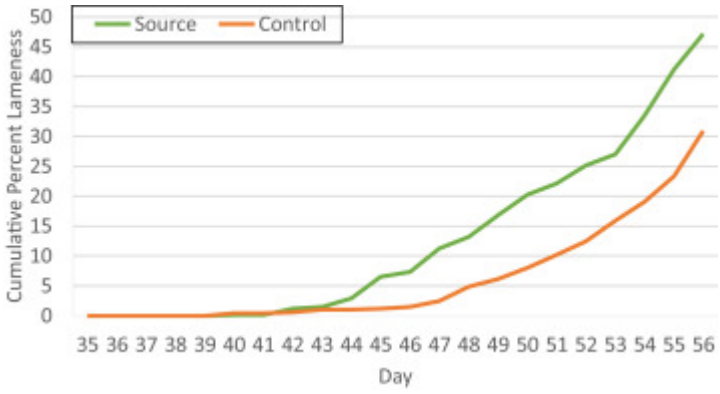

Figure 13. Cumulative percentage lameness for birds raised on litter when challenged with Staphylococcus agnetis908, or unchallenged birds in the same room for experiment 1. All birds were raised on standard wood shavings. Source and control are as described in Table 10.

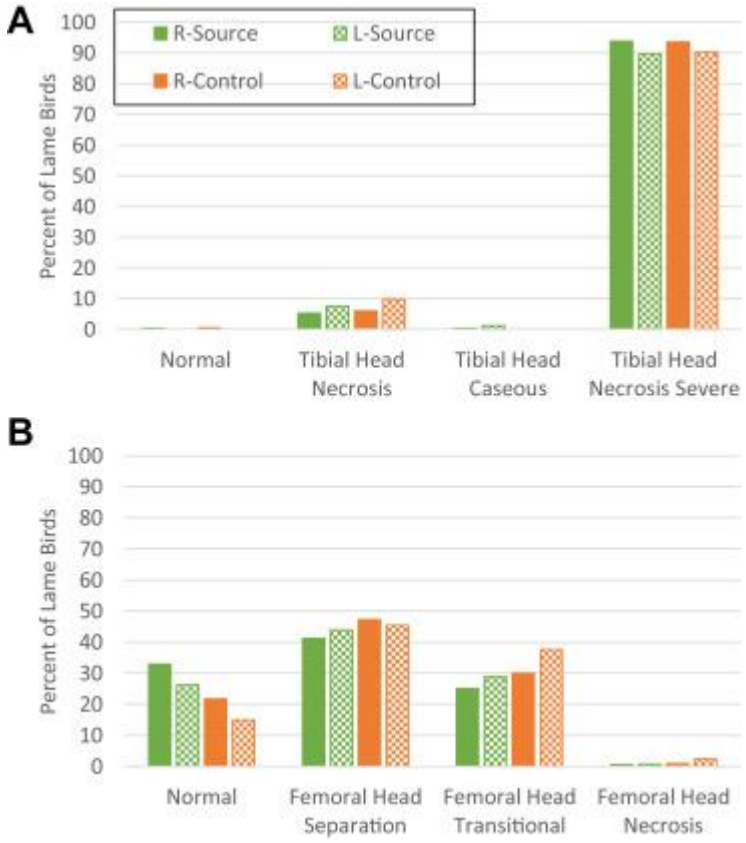

Figure 14. (A) Proximal tibial and (B) femoral head lesion diagnoses for lame birds through the course of experiment 1. Treatments as described in Figure 13 and Table 10. Percentage of lame birds with the indicated BCO lesion is plotted for each treatment. Abbreviations: R, right; L, left; BCO, bacterial chondronecrosis with osteomyelitis.

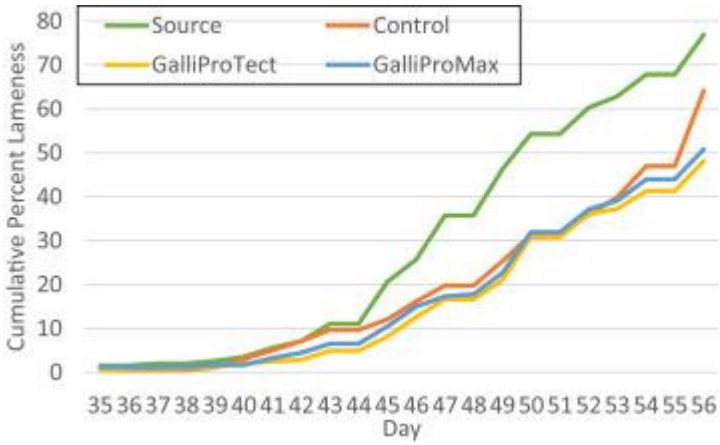

Figure 15. Cumulative percentage lameness for birds raised on litter when challenged with Staphylococcus agnetis 908, or unchallenged birds in the same room with and without probiotics in the feed, in experiment 2. Treatment groups are as described in Table 10.

Table 7. Total lame, percent lame, and dead non-lame, birds for individual pens for each of the treatments through 56 d in experiment 2.

Treatment	Lame bird count/pen						Percentage lame birds/pen						Dead non lame/pen				
Pen	1	2	3	4	5	Avg[1]	1	2	3	4	5	Avg	1	2	3	4	5
Source	32	44	35	42		38.3 ± 2.5	64	88	71	84		77	0	0	1	0	
Control	26	37	36	28		31.8 ± 2.4	52	74	73	56		64	0	0	1	0	
GalliPro Tect	21	21	26	24	27	23.8 ± 1.1	43	42	54	48	54	48	1	0	2	0	0
GalliPro Max	27	31	29	24	15	25.2 ± 2.5	54	65	60	48	30	51	0	2	2	0	0

Average (Avg) ± SEM calculated for total lame excluding dead non-lame.

Table 8. Generalized linear model *P* values comparing different treatments in experiment 2.

Treatment	*P* values comparing treatment groups[1]		
	Control	GalliProTect	GalliProMax
Source	0.003	0.000000001	0.00000008
Control		0.002	0.02
GalliProTect			0.41

Experimental unit is individual bird. Birds that died of causes other than lameness were excluded from the calculations.

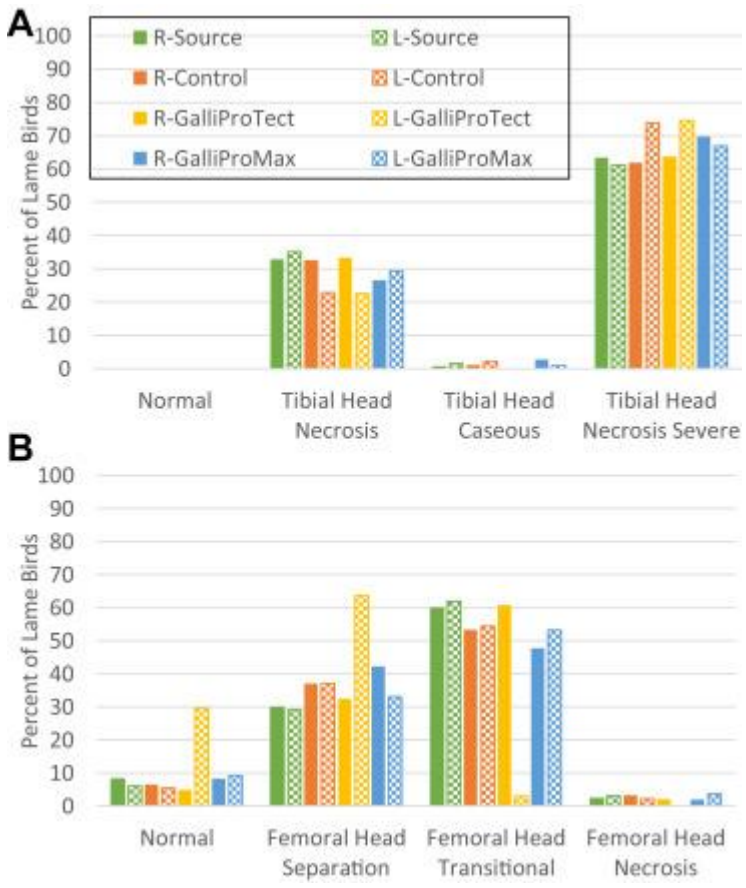

Figure 16. (A) Proximal tibial and (B) femoral head lesion diagnoses for lame birds through the course of experiment 2. Treatments as described in Figure 15 and Table 10. Percentage of

lame birds with the indicated BCO lesion is plotted for each treatment. Abbreviations: R, right; L, left; BCO, bacterial chondronecrosis with osteomyelitis.

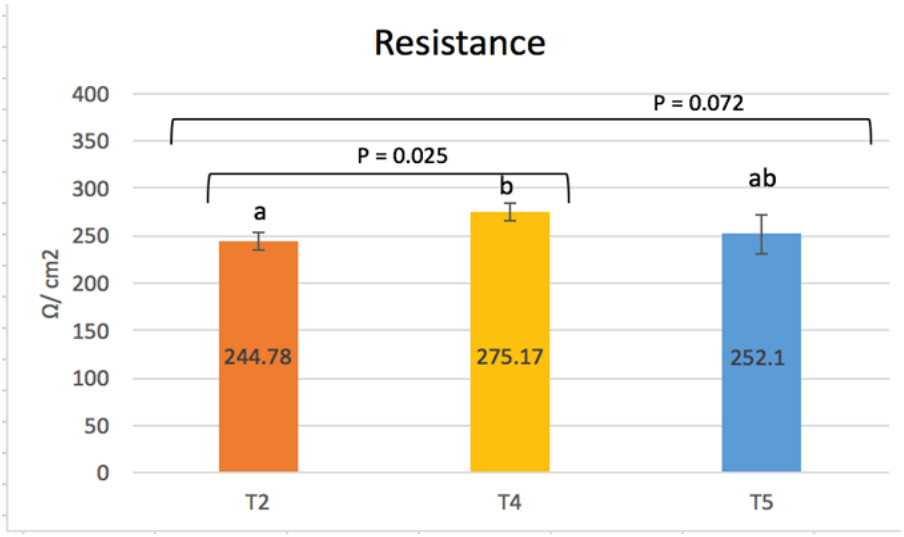

Figure 17. Transepithelial electric resistance values for ileum tissue from apparently healthy birds on day 57 from 3 treatment groups in experiment 2. Values plotted are averages (error bars are SEM) from 10 birds from each treatment. Indicated P-values are relative to control. Where treatments share a letter there is no significant difference at P, 0.05.

Table 9. Relative villus length for the ileum and jejunum for each of 3 treatment groups in experiment 2.

Region	Treatment	N[1]	Villus length (µmol/L)	*P*-value[2]

			Average ± SEM	GalliProTect	GalliProMax
Ileum	Control	137	1,085 ± 19	0.0006	0.000001
Ileum	GalliProTect	116	1,174 ± 19		0.12
Ileum	GalliProMax	139	1,203 ± 15		
Jejunum	Control	118	1,434 ± 27	0.04	0.17
Jejunum	GalliProTect	120	1,380 ± 14		0.0009
Jejunum	GalliProMax	107	1,472 ± 25		

1 Number of separate villus length measurements.

2 *P*-values based on unpaired student T-test.

Table 10. Ruptured villus tip assessment for intestinal pathology for 3 treatment groups in experiment 2.

Treatment	Number of birds	Birds showing ruptured villi[1]	Percent of sections with ruptured villi[1]
Control	10	7	50
GalliProTect	9	5	44
GalliProMax	9	2	14

1 Based on 4 sections from jejunum from each bird.

Table 11. Body weights for apparently healthy birds from each treatment on day 56 in experiment 2.

Treatment	Average BW kg ± SEM[1]	*P*-value[2]

		Control	GalliProTect	GalliProMax
Source	4.40 ± 0.05	0.021	0.17	0.047
Control	4.14 ± 0.07		0.17	0.34
GalliProTect	4.27 ± 0.10			0.28
GalliProMax	4.19 ± 0.10			

1Average from 8 birds.

2*P*-value computed by a T-test comparing the treatment in the first column to each of the other treatments.

CHAPTER 4

The Effectiveness of Synbiotics (PoultryStar™) in Reducing Lameness Incidence in Broilers.

The Effectiveness of Synbiotics (PoultryStar™) in Reducing Lameness Incidence in Broilers.

Disclaimer

I was a member of the team that carried out the project's animal testing during this trial.

UA code: Lameness Experiment #29

Biomin code: FD56

INTRODUCTION

The combination of weak intestinal barriers, bacterial translocation, and bacterial colonization of micro-fractures in fast-growing bones can lead to bacterial chondronecrosis with osteomyelitis (BCO) lameness in broilers. While wire floor models have been extensively used in BCO lameness trials, commercial production facilities use litter floors. The objective of this trial was to determine the effect of flooring types on BCO lameness and intestinal integrity. Commercial symbiotic (PoultryStar® BRO) have been tested in the farm to see if they can lower the incidence of BCO lameness in broiler chickens (Al-Rubaye et al., 2017; Al-Rubaye et al., 2020; Wideman et al., 2014). Pathogenic bacteria (e.g., Staphylococcus spp., Enterococcus spp., and even E. coli) housed in the gut, slowly seep through the intestinal epithelium. Due to the compromised physical barrier, intestinal bacteria in the lumen can pass into the lamina propria, enter the bloodstream, travel to organs, and cause infections, including the joints. There are microfractures in lengthy bone growth plates that allow germs to enter and start destroying the bone. Because of this, the major goal to defend against BCO is by improving gut health and barrier function.

Tight junctions are intercellular adhesion complexes in epithelia and endothelia that control paracellular permeability. It is made up of over 40 proteins. Actin's connection to a tight junction permits it to close and open in response to the environment. It is one of the physical barriers in the innate immune system located in the digestive tract that inhibits luminal antigens or bacteria from entering the mucosa and subsequently reaching the blood stream, resulting in bacterial translocation.

Probiotics and feed supplements have been shown to reduce lameness by 20–25 percent in numerous broiler studies by strengthening tight junctions in the gut and increasing the bactericidal activity of peripheral blood monocytes, two mechanisms that are thought to promote gut integrity (Al-Rubaye et al., 2020). In order to determine whether a reduction in BCO lameness of at least 20% could be achieved in the commercial broiler sector, this study included five treatment groups, a control group, three Biomin probiotics groups, and a wire-flooring group to induce BCO lameness. This reduction in BCO lameness would improve animal well-being and reduce financial losses from BCO lameness. Numerous studies have revealed that the most often isolated bacteria from joint lesions in birds with BCO lameness include Staphylococcus spp., Enterococcus spp., E. coli, and Mycobacterium spp. These findings, which are based on the fact that all of the bacteria mentioned above are members of the intestinal microbiota, imply that bacteria can be transmitted from the gut to joints across the gut barrier, as well as translocation of luminal bacteria to underlying tissue and interior organs.

Materials and methods

Animal housing, care, and treatment

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (# 21066). The trials were conducted in floor pens in building A365W at the University of Arkansas Poultry Research Farm. We flushed carboys and nipple waterers at least two days after removing bacterial biofilms with weak bleach (5%) and a tap water flush prior to the chicks being placed. All birds were on a 23-hour day. Thermoneutral/Optimal brooding and growth temperatures are maintained throughout (32.2 ^{o}C for d1 to d3). And throughout, there is ventilation. 31.1 ^{o}C for d4 to d6, 29.4 ^{o}C for d7 to d10, 26.7 ^{o}C for d11 to d14, and 23.9 ^{o}C). The initial diet consisted of commercial corn and soybean meal. On day d35 (11/02/2020), all birds were transferred to a pelleted commercial starter (crumbles) constituted using corn and soybean meal. The feed was created without the use of meat or animals and meets or exceeds the minimal requirements established by the National Research Council (1994) for all ingredients. *Ad libitum* starter and finisher feeds were provided. Body weights were not recorded, so as not to induce further stress on already stressed birds during the studies. Wire flooring is postulated to induce significant stress (Wideman & Pevzner, 2012; Wideman & Prisby, 2013).

Treatment groups will be the flat wire (CW) (positive control); represents the Probiotic 1 (FA1); represents the Probiotic 2 (FA2); represents the Probiotic 3 (FA3); and fresh litter (CL) negative control (Table 12).

Bird stocks were 840 Cobb500 surplus males from the female fast-feathered breeder chicks from the Fayetteville Hatchery were obtained on day of hatch. Wideman et al. (2012) described a suspended wire flooring setup for 60-chicken enclosures. Bird initial densities were approximately 1.65 ft2/chick in all enclosures. A365W is equipped standard with computer-controlled systems to regulate temperature, light, and ventilation. On day 14 each

pen was culled to a capacity of no more than 50 birds. From day 22 onward, brooms were used to "walk" all the birds daily to check for lame birds. On day 22, lameness was recorded for each pen. Each lame bird was necropsied and scored for BCO lesions as described (Wideman *et al.*, 2012).

Previously published pictures depicted typical proximal femur and tibiae BCO lesions (Wideman & Prisby, 2012; Wideman et al., 2012). To illustrate the progressive nature of BCO, proximal femoral head lesions (FHS, FHT, FHN) and tibial head lesions (THN, THNs, THNc, TD) were classified individually (Wideman et al., 2012). On day 56, representative surviving birds were euthanized and necropsied in order to determine the frequency of subclinical lesions: Proximal femoral head normality; Femoral Head Separation; Femoral Head Transitional Degeneration; Femoral Head Necrosis; Tibial Head Necrosis; Tibial head necrosis severe; Tibial head necrosis caseous and Tibial Dyschondroplasia (Table 13).

Cervical dislocation and necropsy were performed on five birds in each treatment at 56 days of age. Immediately after removing the mesentery and connective tissues from the animals, the jejunum and ileum samples were dissected free of the jejunum and ileum. Segments of the mid-jejunum and mid-ileum were removed and submerged in RNAlater (Sigma Aldrich, St. Louis, MO) immediately after being cleansed with ice-cold PBS (pH 7.4). Finally, they were kept at - 20 °C in order to be studied further A method based on guanidinium isothiocyanate was used to extract RNA. This method was adapted from Chomczynski and Sacchi (Chomczynski, 1987). A modified version of protocol (U Gubler and B J Hoffman, 1983) was used to produce the complementary DNA (cDNA)

H2O to make final volume 20 µl accepting volumes for RNA, primers, and enzymes

2 µl 10x Taq Polymerase buffer

0.2 µl 20 mM dNTPs (all four)

1 µl 20x EvaGreen dye Mix well

4U Taq Polymerase

Mix Well

3) Aliquot to tubes for adding cDNA. Add enough cocktail for one to two extra reactions.

4) Add about 6 µl cDNA for each gene. Mix well. Extra cDNA can be frozen at -20 for use later.

5) Pipet 54 µl for each cDNA mixture into every third well in the qPCR plate, then add 6 µl of 10 mM Forward and Reverse primers. Mix well by trituration with a 50 µl multichannel pipettor so you are mixing multiple mixtures. Then set the multichannel to 19.5 µl and using the same tips pipet to the other two wells for the triplicate reactions. (Dr. Rhoads, Molecular Genetics Laboratory Procedures 2021).

Representative protocol is:

°C	Seconds	Cycling
90	30	Initial Denaturation
90	15	10X
55	15	
72	60	
90	15	30X

55	15	
72	60 than read	
72	180	Final soak
90	15	High Resolution Melt curve
65	180	
75 to 90@ 0.1°C	5 sec than read	

Results

A total of 840 Cobb 500 male chicks were allotted either 2 pens flat-wire (CW), 3 pens fresh litter (CL) and 3 pens on litter for each for the three Biomin probiotics (FA1, FA2, & FA3) studied. Stocking densities were 60 birds per pen from d 0–14 and 50 from d 14–57. Starting on d 21, all birds were walked daily to assess BCO lameness based on resistance to move when motivated. On d 57, all birds were necropsied and BCO lameness assessed. Mortality and BCO lameness percentage were analyzed with the GLIMMIX procedure of SAS. The CW pens were situated closest to the cooling pads to allow airflow from the CW pens to the remaining pens. The purpose of this strategy was to transmit aerosolized germs from CW birds to the litter-housed birds in order to cause BCO lameness. As a result, the CW treatment was omitted from the statistical analysis because it was included solely as a source of aerosolized germs. Daily lameness assessments began on d21, and on d56 representative surviving birds were weighed. We euthanized birds that were clinically lame and assessed femur and tibia bone abnormalities. SAS (9.4 Cary, NC) with the GLIMMIX method was used to analyze treatment effects. When the treatment means were statistically different ($P < 0.05$), the means for separation were calculated. The cumulative percentage of lameness per

treatment during the course of the experiment. After Day 42, the percent lameness for treatments FA1, FA2, and FA3 begins to diverge from the wire-flooring (CW) and control (CL), and then continues to decline to near 30%, with CL finishing at 63%. FA1 and FA2 had nearly similar 38% totals, Biomin products (poultrystar) were tested to see what effect they had on the occurrence of lameness in various treatment groups. FA3 is the most effective at reducing BCO-induced lameness in birds, with a 30-percent occurrence rate. In contrast, CL has a lameness rate of more than 60%. Body weight on d56 and overall FCR were not significantly different between treatments ($P > 0.05$) (Table 15). Overall, the incidence of lameness in FA1, FA2 and FA3 birds (35%) was reduced ($P < 0.05$) when compared to CL birds (65 %). Biomin birds had significantly lower scores for femoral head separation (FHS) and tibial head necrosis (THN) bone lesions ($P < 0.05$) compared to CL birds. Although Bro birds had numerically fewer femoral head transitional degeneration (FHT), femoral head necrosis (FHN), and kinky back (KB) than CL birds, these differences were not statistically significant ($P > 0.05$). In this trial, the CW model was successful in transmitting BCO to CL birds. Biomin supplementation reduced BCO by 46% in this trial when compared to CL birds, indicating that it may be a viable method for broiler lameness reduction (Table 16).

The intestinal barrier is weakened in birds with clinical symptoms such as lameness from chondronecrosis and neurological indications from osteomyelitis (Bacterial Chondronecrosis with Osteomyelitis – Lameness, 2022). Although the mechanism of disruption of tight junctions is unknown, it is apparent that the gut's beneficial bacteria play a role in its regulation. The colonization of the intestines with poultry probiotics can help minimize pathogen translocation. Some research suggests controlling the tight connection with

methionine and cinnamaldehyde, however this is still debated (Bacterial Chondronecrosis with Osteomyelitis – Lameness, 2022).

In the jejunum and ileum, we examined expression of three tight junction proteins: Occludin, claudin-2, and zonula occludens-1. Bacterial subclinical dramatically decreased Occludin, Claudin-2, and ZO-1 gene expression in the ileum, but probiotic administration significantly boosted Occludin, ZO1, and Claudin-2 mRNA expression (Figure 18). Bacterial infection significantly decreased Occludin, ZO1, and claudin-2 gene expression in the jejunum, but probiotic supplementation boosted Occludin gene expression (Figure 19).

Discussion

There is growing evidence that giving chickens a probiotic or a mix of probiotics and prebiotics (symbiotic) reduces the incidence of BCO lameness (Al-Rubaye et al., 2020). Although the probiotics utilized vary, they all improve gut integrity and reduce bacteremia, which affords opportunities for pathogens to colonize bone stress fractures in rapidly expanding bones. Thus, probiotics provided constantly may be an effective method for preventing BCO lameness.

PoultryStar® BRO have been shown to be effective in preventing against and/or lowering pathogen infection in chickens when given in place of antibiotics (Hu. J.Y. et al., 2022). The effectiveness of probiotics can be determined by observing their effect on growth performance, gut permeability, inflammation, and the reduction of pathogenic infection in animals.

Diet, intestinal microbiota, stress, illnesses, infections, toxins, and antibiotics are all known to influence intestinal permeability, which can be influenced by a variety of factors (Kansagra et al., 2003). Increased intestinal permeability in chickens is frequently connected with poor health and performance, bacterial translocation, coccidiosis, immunological activation, and lameness. Increased intestinal permeability in chickens is also associated with lameness (Wideman et al., 2012). The purpose of this study was to investigate the effects of probiotics on reducing or preventing lameness in broiler chickens, as well as the changes in gut barrier function and integrity, by determining the mRNA and gene expression of gut barrier function and Tight junctions related genes in broiler chickens.

TJ proteins have been shown to be critical in regulating intestinal permeability (Groschwitz and Hogan, 2009). It has been shown that barrier function can be measured indirectly in vivo by quantifying mRNA expression, determining phosphorylation, protein folding, and TJ protein localization (Awad et al., 2017).

Probiotic supplements benefit broiler performance by promoting a healthy balance of microorganisms in the digestive tract, improved intestinal integrity, and metabolism (Sugiharto S, 2016).

According to ecological theory, bacterial species diversity relates to gut microbiota stability. The use of multi-strain probiotics in broiler diets may help minimize susceptibility to pathogen invasion and intestinal inflammation, while also improving intestinal absorption and growth performance (Li Y. et al., 2016).

The animal's initial line of defense against infections is provided by the intestinal barrier function. Epithelial cells are protected from macromolecular translocation by proteins called

tight junctions, which operate as a fence. Occludin and Claudin genes were downregulated in the intestines of broiler chickens after infection.

The permeability of the intestinal lumen was found to be linked with tight junction proteins. Pathogens' breakdown of the intestinal barrier enabled macromolecules from the intestinal lumen, such as antigens, bacterial toxins, and infections, to enter the circulation (Wang W et al., 2016).

Table 12. Treatment group descriptions for the Biomin experiment 29

Treatment	Description	Pens
CW	Diet 1 (regular diet with no probiotics): Wire-Flooring challenge pens	1, 8
T2	Diet 2 (FA1)	2, 7, 12
T3	Diet 3 (FA2)	3, 5, 14
T4	Diet 4 (FA3)	4, 10, 13
CL	Diet 1 (regular diet with no probiotics): control group	6, 9, 11

Table 13: Experimental protocol for the Biomin experiment 29

Age	Date	Day	Temp. °F	Comments
1	01-Oct	Thursday	90F	Place 60 chicks per pen. All pens on Starter crumbles.
14	14-Oct	Wednesday		-Cull to 55 birds per pen
22	22-Oct	Thursday		Begin recording all deaths, lame and infirmed.
30	25-Oct	Tuesday		• Birds in pens 4-6, 10, 15-17, 21 and 22 will be culled to 40 bird/pen. Ten birds from pens 1 & 13 will be added into pens 4-6, 10, 15-17, 21 and 22.
35	05-Nov	Thursday		Switch all pens to finisher pellets.
56	25-Nov	Wednesday		Necropsy 10 birds per treatment group for lameness category, but collect: proximal tibial and femoral heads from 2 lame birds/pen for bone microbiology culture. Histopathology samples from same bones will be saved for possible histopathology work if funding is provided by Biomin.

Gene expression.

Compared to the control group (Figure 18 and 19), symbiotic increased the amount of mRNA that expressed ZO-1 and decreased the amount of mRNA that expressed CLDN2 in both the ileum and the jejunum. ZO-1 is also found in the jejunum mucosa (P > 005). But the treatments did not change the amount of OCLN mRNA in the intestinal mucosa (P > 005) (Table 14).

Table 14. Sequences for real-time PCR primers

Genes	Gene bank ID	Primer sequence, sense/antisense	Length (bp)	Efficiency (%)
TBP	NM_001396190.1	CAGGGCATGAAACGTGG CAGAGCAAAGGGGAAGGAT	402	92.92
OCLN	NM_205128.1	CCGTAACCCCGAGTTGGAT ATTGAGGCGGTCGTTGATG	214	97
CLDN2	NM_001277622.1	CCTGCTCACCCTCATTGGAG GCTGAACTCACTCTTGGGCT	145	101
ZO-1	XM_413773.4	TGTAGCCACAGCAAGAGGTG CTGGAATGGCTCCTTGTGGT	159	102

OCLN, occludin; CLDN2, claudin-2; ZO-1, zonula occludens-1. (Chen, 2018)

Table 15. Total clinically lame and mortalities (includes lameness) for birds raised on wire-floor (CW), fresh litter (FL), and fresh litter supplemented with PoultryStar® BRO (BRO) over a 57-day study[1].

Treatment	Clinically lame, %	Total mortality, %
CW	76.7	97.4
FL	64.7	87.3

BRO	35.0	52.0
SEM	3.6	3.2
P-value	0.005	0.003

[1]The SEM and *P*-value represents standard error of the means and statistical significance of the difference, respectively, between FL and BRO; CW is not included in the analyses and listed for reference only.

Table 16. Body weight gain (d 0 – 56) and FCR for birds raised fresh litter (FL) and birds raised on fresh litter fed PoultryStar® BRO (BRO)[1].

Treatment	Body weight gain, kg	FCR
FL	3.55	2.22
BRO	3.91	2.10
SEM	0.69	0.08
P-value	0.735	0.370

[1]The SEM and *P*-value represents standard error of the means and statistical significance of the difference, respectively. The FCR was calculated based on projected performance with 60 birds per pen.

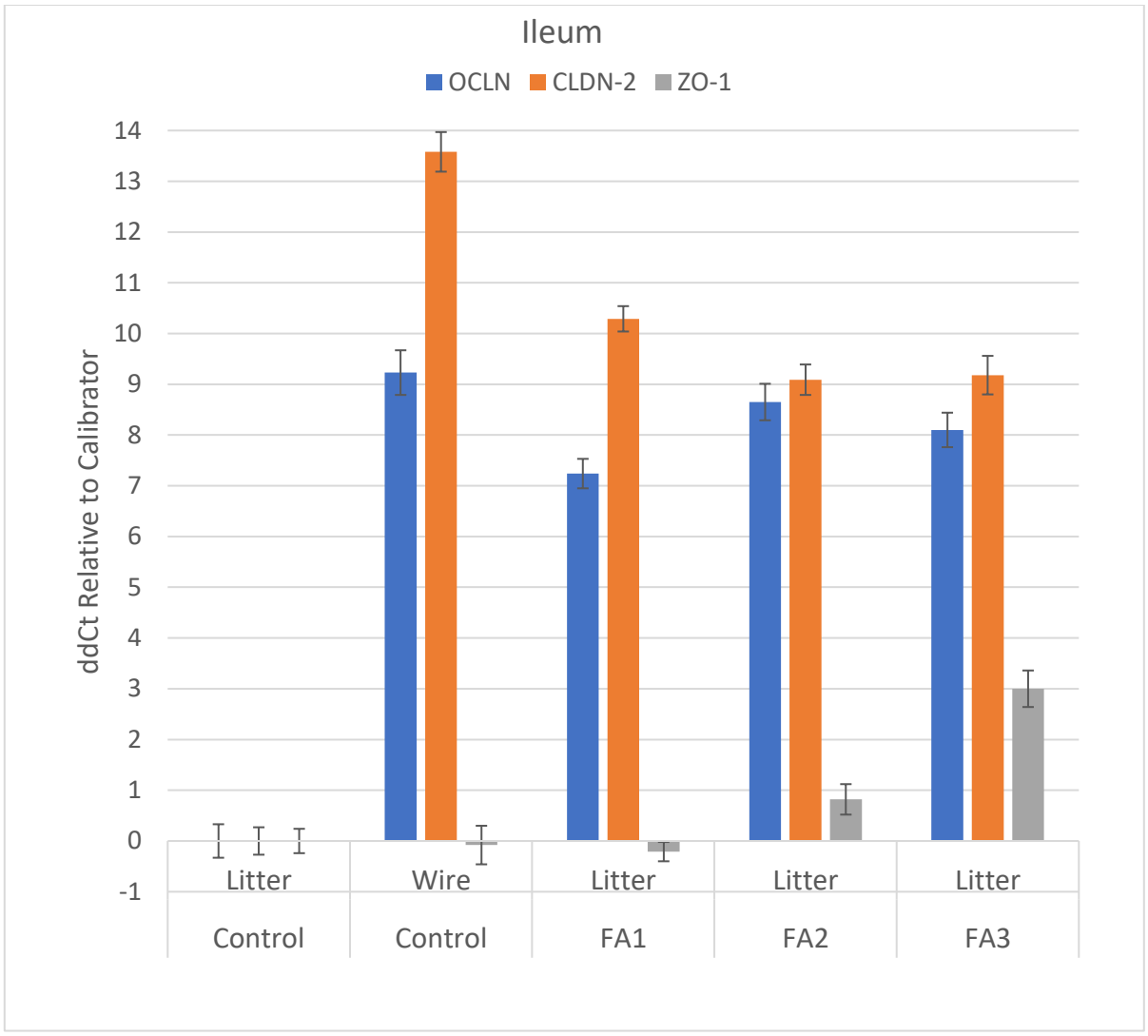

Figure 18. Relative gene expression was calculated using the 2-ΔΔCt method in ileum.

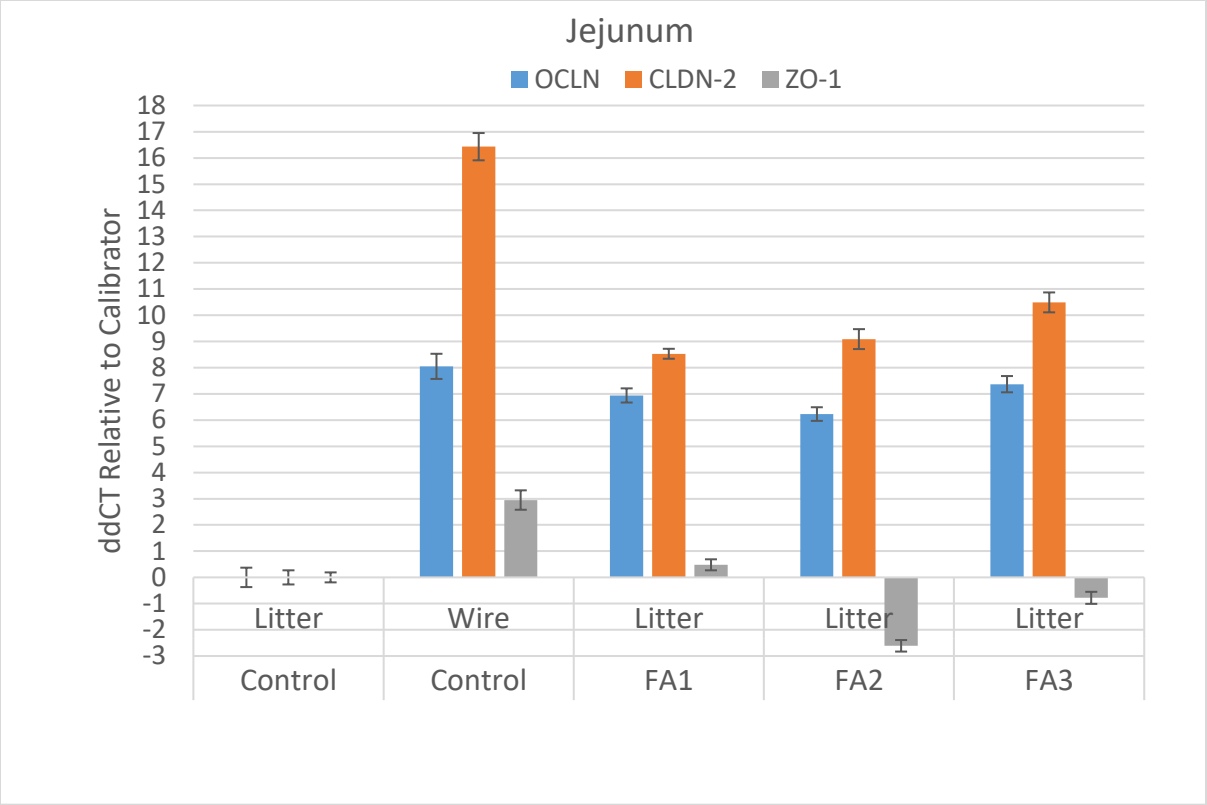

Figure 19. Relative gene expression was calculated using the 2-ΔΔCt method in Jejunum using litter as the reference.

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